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14. ABSTRACT

A single intra-tumoral injection of IL-12 + GM-CSF-encapsulated microspheres promoted the suppression of primary tumor growth, prevented the development of additional tumors and extended survival in the her2-neu transgenic FVB/neuN murine mammary tumor model. Induction of a long-lived, systemic, tumor-specific immune response was demonstrated in adoptive cell transfer studies, cytotoxic T-cell assays, in vivo lymphocyte subset depletion studies and analysis of serum anti-tumor antibody levels. Repeated treatment was more effective than single treatment in inducing complete tumor regression, secondary tumor suppression and enhancing survival, however long-term cures were not obtained. Chemoimmunotherapy did not improve long-term survival. Further analysis of tumor-infiltrating T-cells demonstrated that repeated therapy resulted in the enhancement of T-suppressor cell activity and the loss of tumor-specific cytotoxic T-lymphocytes.

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Table of Contents

INTRODUCTION

This proposal aims to test the efficacy of a novel cytokine-based tumor vaccination strategy in the FVB/neuN murine spontaneous tumor model. Our approach involves a single intra-tumoral injection of Interleukin-12 (IL-12) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF)-loaded biodegradable microspheres to induce anti-tumor immunity. In the first year of studies the effect of this *in situ* vaccination strategy on the growth of established primary tumors, the suppression of secondary tumor development and the induction of long-term, systemic anti-tumor is investigated. The second year studies involve a more detailed analysis of the long-term anti-tumor immunity and the evaluation of this treatment strategy in a surgical model where the primary tumors are resected after vaccination. Finally, in the third year, the efficacy of combined treatment with IL-12 + GM-CSF microspheres, surgery and chemotherapy is evaluated in the surgical model.

BODY

Summary.

The tasks for year 1 were:

1. To monitor the FVB/neuN mice for tumor development and to characterize the model.
2. To monitor the effect of microsphere treatment on the growth of primary tumors, the development of secondary tumors and survival.
3. To evaluate the effect of microsphere dosage and vaccination schedule on the growth of primary tumors, secondary tumor development and survival.

The tasks for year 2 were:

1. To monitor the effect of IL-12 + GM-CSF microsphere treatment on the development of long-term systemic anti-tumor immunity by cytotoxic T-cell assays and by adoptive transfer of splenocytes from vaccinated mice to naïve mice.
2. To determine the efficacy of combining immunetherapy with surgery for the treatment of spontaneous tumors in a clinically relevant embodiment of the FVB/neuN model.
3. To evaluate the efficacy of repeated vaccinations with tumor cells and cytokine-encapsulated microspheres on the growth of primary tumors, secondary tumor development and survival.

The tasks for year 3 were:

1. To perform in vivo lymphocyte subset depletion experiments prior to vaccination to determine which subsets are involved in the priming of the anti-tumor immune response.
2. To treat tumor-bearing mice with chemotherapy to determine whether chemoimmunotherapy improves long-term tumor suppression.
3. Monitor post-vaccination serum cytokine levels in mice treated with chemoimmunotherapy to determine whether immune responses are enhanced after chemotherapy.
4. Monitor the ability of adoptively transferred splenocytes from mice above to suppress tumor growth in naïve mice.

All tasks have been completed except tasks 3 and 4 for Year 3 due to the finding that chemoimmunotherapy did not improve long-term survival compared to immunotherapy alone. However, further analysis of the post-therapy immune responses demonstrated in Year 2 tasks 1-3 and Year 3 task 1 resulted in novel findings which were published recently (references 1 and 2 below).

Results.

1. Development of spontaneous breast carcinomas in the FVB/neuN mice. See year 1 report.

2. Preparation and characterization of the IL-12 and GM-CSF-encapsulated microsphere formulations. See year 1 report.

3. Treatment of established breast tumors with IL-12 + GM-CSF microspheres and monitoring of primary tumor growth and secondary tumor development. See year 2 report and reference 1 (appendix).

4. Effect of microsphere dose on tumor suppression. See year 2 report and reference 1 (appendix).

5. Effect of treatment frequency on tumor suppression. See year 2 report and reference 1 (appendix).

6. Development of long-term systemic anti-tumor immunity in vaccinated mice. See year 1 report and reference 2 (appendix).

7. Surgical resection of treated tumors does not improve tumor recurrence rate and adversely affects secondary tumor suppression. See year 2 report.

8. Repeated vaccinations with mixtures of tumor cell suspensions and microspheres. See year 3 report.

9. Both CD4+ and CD8+ T-cells are critical to the post-therapy tumor regression.

   The role of CD4+ and CD8+ T-cells in post-therapy tumor suppression was determined in in vivo subset depletion studies. Mice were depleted of either CD4+, CD8+ or both subsets by administration of antibodies prior to vaccination and tumor growth was monitored in control (irrelevant antibody) and depletion groups. The results demonstrated that both CD4+ and CD8+ T-cells were critical to therapy-mediated tumor regression. These results are published in reference 1 (see appendix).

10. Chemoimmunotherapy does not enhance tumor eradication in comparison to immunotherapy alone.

    To determine whether administration of low dose cyclophosphamide in combination with immune therapy could enhance long-term tumor suppression (Aim 3, tasks 2-4), mice were treated with 200 mg/kg of cyclophosphamide (CTX) at different times before or after vaccination and survival was monitored. The results shown in Figure 1 demonstrate that combining immune therapy with CTX did not enhance long-term survival.

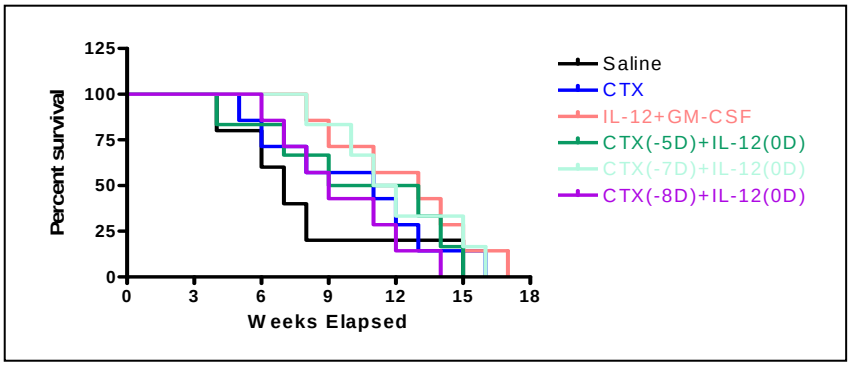

Figure 1. Long-term survival analysis of mice treated with CTX + IL-12/GM-CSF vaccination. Mice were monitored until primary tumors reached a size of 100-200 mm^3. They were then either administered CTX at different times prior to vaccination. Vaccination was on day 0. Control mice received either no treatment, (saline), CTX alone or IL-12 + GM-CSF alone. Tumor growth was monitored until the tumors reached 15 mm in diameter at

which time the mice were sacrificed. The differences between CTX+ IL-12/GM-CSF and IL-12 + GM-CSF alone were not statistically significant by Log-Rank analysis ($p > 0.05$, $n = 8$ per group).

Administration of CTX on days other than shown above (-3, +3, +7) did not enhance survival (data not shown). Since these results were negative, Tasks 3 and 4 were not pursued.

11. Repeated vaccination of recurring primary tumors with IL-12/GM-CSF microspheres enhances tumor suppression but the effect is transient and becomes progressively inefficient.

    Since the anti-tumor activity of a single treatment was found to be transient, the efficacy of repeated treatments (every 3 weeks) in achieving long-term tumor regression was tested. These studies demonstrated that while additional treatments enhanced short-term tumor regression, it did not improve long-term survival. Furthermore, treatment became increasingly ineffective with repeated administration of IL-12/GM-CSF microspheres. These data are presented in reference 1, see Appendix).

12. Treatment induces anti-tumor CD8+ T-cell activation and the purge of T-suppressor cells but these effects are transient.

    To determine the mechanisms underlying the transient nature of tumor regression and the failure of chronic therapy, intra-tumoral T-cell activity was monitored by real-time PCR. Quantitative analysis of immune activation and suppression markers in fine needle aspirates (FNA) of tumors by real-time PCR demonstrated that treatment initially induced the activation of a CD8+ T-cell response and the concurrent loss of tumor-associated T-suppressor cells. However this reversal of intra-tumoral immune suppression was transient since T-suppressor cell activity rebounded rapidly within 7 days of treatment. These findings were reported in a recent publication (reference 2, see Appendix).

13. Repeated treatment restores CD8+ T-effector activity and T-suppressor purge, however the reversal of immune suppression is progressively inefficient.

    Long-term monitoring of intra-tumoral T-cell activity during chronic treatment demonstrated that repeated treatment was able to resurrect immune activation but the efficacy of recovery became progressively weaker. In addition, the T-suppressor rebound intensified with each additional treatment. These data are presented in our recent article (reference 2, see Appendix).

14. Repeated treatment results in the eventual loss of anti-tumor CD8+ T-cells.

Analysis of her-2/neu-specific CD8+ T-cells by tetramer analysis demonstrated that the initial treatment induced a potent infiltration of tumors with tumor-specific CTL on day 7 after treatment. These CTL were shown to be highly cytotoxic to tumors. However, repeated treatment resulted in the loss of the anti-tumor CTL. The remaining CTLs, although few in numbers, were still functional with regard to cytokine production. These data are also presented in the second manuscript.

KEY RESEARCH ACCOMPLISHMENTS

- Chronic therapy resulted in superior anti-tumor immunity, however this did not result in a significant improvement to long-term survival.
- Primary tumor suppression/eradication was shown to be mediated by both T- and NK/NKT cells.
- Analysis of T-cell infiltration kinetics into the tumors demonstrated that the T-cells successfully infiltrated tumors after treatment however the anti-tumor T-cell response peaked at day 7 post-treatment and

gradually diminished afterwards resulting in tumor recurrence.

- The development of a potent anti-tumor B-cell response was demonstrated in treated mice. However, this response required multiple treatments to reach significant levels.
- Treatment reverses intra-tumoral immune suppression by inducing tumor-specific CD8+ T-cells and by purging T-suppressor cells.
- The reversal of intra-tumoral immune suppression is transient as T-suppressor cells rebound quickly. Repeated therapy can resurrect immune activity but T-suppressors rebound becomes progressively stronger and repeated therapy eventually fails.
- The progressive enhancement of T-suppressor cell rebound is paralleled by loss of tumor-specific CD8+ T-cells during chronic therapy.

REPORTABLE OUTCOMES

The findings above were reported at 2 international meetings and were recently published in two separate scientific journals.

Publications:

1. Nair RE, Jong YS, Jones SA, Sharma A, Mathiowitz E and **Egilmez, NK**. 2006. IL-12 + GM-CSF Microsphere therapy induces eradication of advanced spontaneous tumors in Her-2/neu transgenic mice but fails to achieve long-term cure due to the inability to maintain effector T-cell activity. J Immunother 29(1):10-20.

2. Nair RE, Kilinc MO, Jones SA and **Egilmez, NK**. 2006. Chronic immune therapy induces a progressive increase in intra-tumoral T-suppressor activity and a concurrent loss of tumor-specific CD8+ T-effectors in her-2/neu transgenic mice bearing advanced spontaneous tumors. J. Immunol. 176(12):7325-34.

Meeting Abstracts:

1. International Society of Biological Therapy Meeting in San Fransisco, CA (November 2004). This abstract was chosen for oral presentation.

2. The Keystone Meeting on Basic Aspects of Tumor Immunology in Keystone, CO (March 2005).

PERSONNEL

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CONCLUSIONS

The results reported here demonstrate that treatment with IL-12 + GM-CSF microspheres induced both innate (NK-cell) and adaptive (T- and B-cell) anti-tumor responses which successfully infiltrated and suppressed tumors. However the anti-tumor immunity (primarily the T-cell response) peaked by day 7 post-therapy and then diminished, resulting in transient tumor suppression. Repeated treatments improved tumor suppression,

however complete long-term cure could not be achieved. Combining low-dose chemotherapy with chronic immune-therapy did not improve long-term survival. Further analysis of intra-tumoral immune activity in the chronic therapy setting demonstrated that treatment achieved tumor regression by inducing anti-tumor CD8+ T-cells and via the elimination of intra-tumoral T-suppressor cells. However the reversal of immune suppression was transient as T-suppressor cells rebounded rapidly. Repeated therapy was increasingly inefficient as the T-suppressor rebound became progressively stronger and anti-tumor CD8+ T-cells were lost. These findings underline the critical role of T-suppressor cells in the neutralization of therapy-induced anti-tumor T-cell activity. Strategies combining the blocking of T-suppressor cells with vaccination protocols are likely to be more successful in achieving long-term tumor eradication.

APPENDIX

The two publications listed above are attached.

IL-12 + GM-CSF Microsphere Therapy Induces Eradication of Advanced Spontaneous Tumors in her-2/neu Transgenic Mice But Fails to Achieve Long-Term Cure Due to the Inability to Maintain Effector T-Cell Activity

Raji E. Nair, Yong S. Jong,† Stacy A. Jones,* Atima Sharma,* Edith Mathiowitz,† and Nejat K. Egilmez**

Summary: A single intratumoral injection of interleukin-12 and granulocyte–macrophage colony-stimulating factor–encapsulated microspheres induced the regression of advanced spontaneous mammary tumors, suppressed additional tumor development, and enhanced survival in her-2/neu transgenic mice. Posttherapy tumor eradication was dependent on both $CD4^+$ and $CD8^+$ T cells and correlated with the tumor infiltration kinetics of a transient effector T-cell response. Upon long-term monitoring, tumor regression was found to be temporary, and disease-free survival was not achieved despite the development of systemic anti-tumor cytotoxic T-cell memory and antibody responses. Repeated immunization of mice enhanced short-term tumor suppression, resulting in the complete regression of primary tumors in up to 40% of the mice, but did not improve long-term survival owing to recurrence. The failure of chronic therapy to achieve complete cure was associated with an inability to maintain the intensity of the posttherapy effector T-cell response in this model.

Key Words: cancer vaccines, cytokines, adjuvants, immunotherapy, tumor models

(*J Immunother* 2006;29:10–20)

It is now well established that numerous immune therapy protocols can achieve the eradication of small transplantable mouse tumors in preclinical studies.[1] On the other hand, the same protocols are rarely effective in the clinical setting.[2] Although explanations for the observed discrepancies between preclinical and clinical results have been offered, a full understanding of the lack of clinical efficacy is yet to be established.[3] One major critique of preclinical studies involves the use of transplantable murine tumor models, which may not accurately represent the biology of spontaneously arising, autochthonous human tumors.[4–7] An alternative approach is to use genetically modified mice that develop tumors spontaneously.[4,8] Tumors that arise in these mice reflect the physiology of human tumors more accurately and thus potentially represent a superior alternative to transplantable tumor models for the evaluation of immunotherapy strategies.

One such model involves mice that are transgenic for the rat proto-oncogene her-2/neu driven by the Murine Mammary Tumor Virus (MMTV) promoter.[9] These mice develop spontaneous multifocal mammary carcinomas that metastasize to the lungs as tumors progress.[10] The pathology and the molecular signature of these carcinomas have been shown to be similar to naturally occurring her-2/neu positive human mammary cancers.[11,12] As these mice are embryonal transgenics, her2/neu represents a "self"-antigen, overexpression of which results in tumor development, analogous to the human situation. Although these mice are tolerogenic to her-2/neu, immune manipulation can result in breaking of tolerance.[13–15] These characteristics make her-2/neu transgenic mice a highly attractive model for evaluation of immunotherapy strategies because the primary goal of immune-based cancer therapy in the clinic is to break tolerance to "self" tumor antigens. A number of approaches involving protein/DNA vaccination,[13–15] cytokine-based therapies,[16] as well as chemo-immunotherapy[17] have been tested in her-2/neu transgenic mice with varying degrees of success. The most successful results have been obtained in prophylactic studies that were designed to prevent disease development in young tumor-free mice.[13–16] Therapeutic studies in this model have generally been limited to the treatment of subcutaneous tumors that are induced by injection of her-2/neu-expressing mammary tumor cell lines.[15,17,18] Regression of microscopic spontaneous mammary carcinomas after electroporation with a DNA vaccine was reported recently.[19] However, complete regression of advanced spontaneous mammary carcinomas has not been achieved in her-2/neu transgenic mice.

We previously demonstrated that the local and sustained release of interleukin-12 (IL-12) and granulocyte–macrophage colony-stimulating factor (GM-CSF) from biodegradable microspheres to the microenvironment of a single tumor nodule (in situ tumor vaccination) promotes the suppression of established primary tumors, the development of systemic

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anti-tumor immunity, and the complete eradication of disseminated micrometastatic disease in a transplantable tumor model.[20,21] Here, we demonstrate that in situ vaccination with IL-12 + GM-CSF microspheres also promotes the regression of established spontaneous primary tumors, induces the development of systemic anti-tumor immunity, and enhances survival in her-2/neu transgenic mice. However, in contrast to the results obtained in transplantable tumor models, tumor regression is transient and complete long-term cure is not achieved in her-2/neu transgenic mice even when therapy is administered repeatedly. Clinical implications of these results are discussed.

MATERIALS AND METHODS

Mice

Female mice, FVB/N, or FVB/N-TgN^{MMTVneu}202Mul (FVBneuN) at 6–8 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME, USA). The transgenic mice were shipped monthly (25/month) from Jackson Laboratories where stock breeders are routinely validated for her-2/neu homozygosity. Mice were maintained in microisolation cages (Laboratory Products, Federalsburg, MA, USA) under specific pathogen-free conditions. All studies were approved by the Institutional Animal Care and Use Committee of the University of Louisville.

Cytokines and Microspheres

Recombinant murine IL-12 (2.7×10^6 U/mg) was a gift from Wyeth, Inc. (Andover, MA, USA). Recombinant murine GM-CSF (5×10^6 U/mg) was purchased from Peprotech Inc., (Rocky Hill, NJ, USA). A phase inversion nanoencapsulation technique was used for encapsulation of cytokines as described previously.[20] In brief, bovine serum albumin (BSA; radioimmunoassay grade; Sigma Chemical Co., St. Louis, MO, USA), Polylactic Acid (PLA) (M_r 24,000 and M_r 2,000, 1:1 [wt/wt]; Birmingham Polymers, Birmingham, AL, USA), and recombinant cytokine in methylene chloride (Fisher, Pittsburgh, PA, USA) were rapidly poured into petroleum ether (Fisher) for formation of microspheres. Microspheres were filtered and lyophilized overnight for complete removal of solvent. Three formulations containing 1% BSA (wt/wt) were produced: control (no cytokines), murine IL-12 (2.5 μg [67,500 U]/mg PLA), and murine GM-CSF (2.5 μg [12,500 U]/mg PLA). Scanning electron micrographs demonstrated that the microspheres were 0.1–5 μm in diameter and were easily injectable with a 28.5-gauge needle.

Microsphere Treatments, Monitoring of Tumor Growth, and Survival

Mice were monitored for tumor development by palpation twice a week. All mice developed tumors between 175 and 330 days of age. Treatment was administered with a single intratumoral injection of microspheres when tumors reached about 100–200 mm^3 in size (tumor volume was determined according to the formula $A \times B^2/2$, where A is the longer and B is the shorter perpendicular dimension of the tumor). Experimental groups received IL-12 + GM-CSF–encapsulated microspheres (8 mg of each) suspended in 150 μL of hydration buffer (1% hydroxypropylmethylcellulose [DOW Co., Midlands, MI, USA] and 1% Pluronic F127 [Sigma] in phosphate-buffered saline [PBS], pH 7.2). Control mice received 16 mg of control microspheres in 150 μL of hydration buffer. Both the growth of the primary tumor and the appearance of additional tumors were monitored by palpation twice a week. Mice were killed when the largest diameter of the tumor reached 15 mm.

Histology/Immunohistochemistry

Serial 5-μm-thick sections were prepared from formalin-fixed paraffin-embedded tumor tissue and stained either with the rat anti-human CD3 antibody (catalog no. MCA1477; SeroTec, Raleigh, NC, USA), which cross-reacts with mouse CD3, or a nonspecific isotype-matched (IgG_1) rat antibody (catalog no. 02-918; Zymed, San Francisco, CA, USA) as described.[21] The samples were then incubated with the biotinylated secondary goat anti-rat IgG antibody (catalog no. 554014; Pharmingen, San Diego, CA, USA) followed by avidin–biotin–complex Elite reagent (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine as substrate. Slides were counterstained with hematoxylin, dehydrated, and cover-slipped for light microscope evaluation. Digital images were obtained using a Nikon Eclipse E400 microscope equipped for digital imaging with the DS-L1 digital sight camera system (Nikon Instruments, West Chester, OH, USA). Images were obtained at magnifications of 40×, 200×, and 400×.

In Vivo Lymphocyte Subset Depletions

In vivo depletion of $CD4^+$ and $CD8^+$ T lymphocytes was achieved by repeated injections of purified anti-mouse CD4 (clone GK1.5; ATCC, Manassas, VA, USA) and anti-mouse CD8 (clone 53-6.72; ATCC) monoclonal antibodies, respectively. Starting 1 day prior to vaccination, the mice were injected with anti-CD4 (50 μg in 200 μL of PBS every 7 days for a total of five injections) and anti-CD8 (50 μg in 200 μL of PBS every 4 days for a total of five injections) intraperitoneally. Control mice received nonspecific rat IgG (50 μg per injection; BD Pharmingen, San Diego, CA, USA) every 7 days for a total of five injections. Flow cytometric analysis of splenocytes demonstrated that the above dose and injection schedules for each antibody resulted in the depletion of >98% of targeted lymphocyte subsets for at least 3 weeks (data not shown).

Adoptive Transfer Studies

Splenocytes were isolated from donors 3 weeks after microsphere treatment (control or IL-12 + GM-CSF). Mice were killed by CO_2 inhalation, spleens were removed, and single-cell suspensions were prepared mechanically by teasing the spleen with the blunt end of the plunger of a 10-mL plastic syringe in a sterile Petri dish containing 3 mL of culture medium (Dulbecco Modified Eagle Medium [DMEM] with F12 salts + 10% fetal calf serum [FCS]). The resultant cell suspensions were filtered through a 70-μm cell strainer (Falcon 2340; Becton Dickinson, Orlando, FL, USA), red blood cells were lysed by brief incubation with 0.14 M ammonium chloride solution, and cell debris was removed by Ficoll-Paque PLUS (Amersham Biosciences) centrifugation.

Cells were washed in PBS and resuspended at 1×10^7 viable cells/mL. Splenocytes (pooled from five donors per experiment) were injected intraperitoneally to tumor-free, age-matched recipients (4×10^7 in 0.2 mL of PBS per mouse).

Cytotoxic T-Lymphocyte Assay

Tumor-bearing mice were killed 21 days after treatment with control or IL-12 + GM-CSF microspheres, and the tumors as well as the spleens were removed. Single-cell suspensions were prepared from each tumor by enzymatic disaggregation as previously described.[22] $CD3^+$ T cells were isolated from individual spleens using the murine $CD3^+$ T-cell enrichment columns (R&D, Minneapolis, MN, USA) by high negative selection as per manufacturer's instructions. In brief, 5×10^7 cells were suspended in column binding buffer, loaded onto the column, and incubated at room temperature for 10 minutes. The unbound cells were eluted from the column, washed three times with PBS, and suspended in 1 mL of DMEM/F12 + 10% FCS. The purity of the preparations was confirmed by flow cytometry analysis (>90%; data not shown). The ability of the column-purified $CD3^+$ T cells (effector) to kill autologous tumor cells (target) was determined for each mouse by quantitative measurements of lactate dehydrogenase (LDH) release using CytoTox96 nonradioactive cytotoxicity assay kits (Promega Corp., Madison, WI, USA) as recommended by the manufacturer. Assays were performed at effector/target ratios of 10:1, 5:1, 2.5:1, and 1.25:1, in a final volume of 100 μL with 4×10^4 target cells/well. After a 4-hour incubation at 37°C, 50 μL of the culture medium was collected to assess the amount of LDH. Percentage lysis was calculated according to manufacturer's instructions.

Serum Interferon-γ Levels

A double-sandwich enzyme-linked immunosorbent assay (ELISA) method was used to quantify the levels of interferon-γ (IFNγ) in duplicate titrations of serum samples with a commercial ELISA kit in accordance with the instructions of the manufacturer (Pierce Endogen, Rockford, IL, USA).

Analysis of Serum Anti-Tumor Antibody Levels

Blood was collected from isofluorane-anesthetized mice by orbital sinus bleeding, and sera were stored frozen at −80°C. Mice were killed at the conclusion of the experiment, and single-cell suspensions were prepared from tumors as above. Analysis of serum anti-tumor antibodies and their ability to mediate complement deposition were performed essentially as described,[23] with the following modifications. For antibody binding to tumor cells, serum samples were thawed rapidly at 37 °C, diluted (1:32) with DMEM/F12, mixed 1:1 with 0.1 mL of tumor cell suspension (1×10^6/mL in warm DMEM/F12), and incubated at 37°C for 45 minutes. The cells were then pelleted, washed with PBS, and incubated with fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse IgG (catalog no. GGHL-90F1; Immunology Consultants Laboratory, Newberg, OR, USA) in the dark at 4°C for 30 minutes. For complement deposition, the antibody–cell complexes were washed twice with PBS containing 1% BSA, resuspended in 0.2 mL of naive syngeneic mouse serum (diluted 1:1 with DMEM/F12) as the source of complement, and incubated for 15 minutes at 37°C. FITC-conjugated goat anti-mouse C3 (catalog no. 55500; MP Biomedicals, Irvine, CA, USA) was added and allowed to bind as above. Cells were then washed twice with PBS and analyzed (10,000 events) using a fluorescence-activated cell sorter Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). Mouse IgG1-FITC isotype control antibodies (Becton Dickinson, no. 349041) were used to correct for background fluorescence. Immunoglobulin (Ig) subclass analysis was carried out using the same protocol (without complement), except sera from individual mice were analyzed after three treatments with microspheres. Secondary FITC-conjugated monoclonal antibodies directed against Ig subclasses were as follows: anti-mouse IgG1 (clone A85-1), anti-mouse IgG2a (clone R19-15), anti-mouse IgG2b (clone R12-3), anti-mouse IgG3 (clone R40-82), anti-mouse IgM (clone R12-3), anti-mouse IgA (clone C10-3), and anti-mouse IgE (clone R35-72). All antibodies were purchased from BD Pharmingen (San Diego, CA, USA).

RESULTS

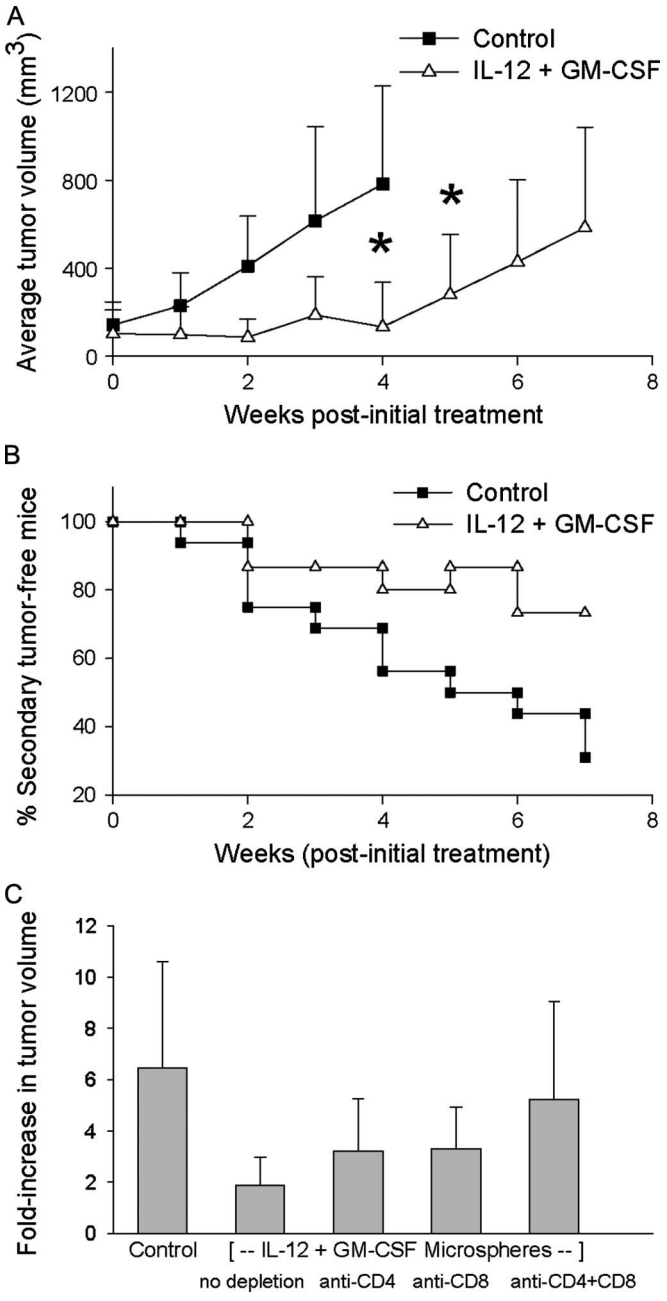
Intratumoral Injection of IL-12 + GM-CSF Microspheres Induces Transient Eradication of Advanced Tumors, Which Correlates With Tumor Infiltration Kinetics of an Effector T-Cell Response

In initial studies, we determined whether in situ therapy with IL-12 + GM-CSF microspheres represents an effective strategy for the treatment of advanced spontaneous mammary adenocarcinomas in FVBneuN mice. Mice were monitored for tumor development and were treated with a single intratumoral injection of IL-12 + GM-CSF microspheres when the primary tumor reached about 100–200 mm^3 in size. The mice were then monitored for the growth and/or regression of the treated primary lesion as well as for the development of additional independent "secondary" tumors as these mice develop multifocal mammary tumors with age. The growth of the treated primary tumor was suppressed in all animals, with 46% of the mice displaying partial (≥50% decrease in the sum of two perpendicular diameters of the tumor) and 13% of the mice achieving complete tumor regression (complete disappearance of the tumor for at least 1 week) by week 4 post therapy (Fig. 1A). In addition, a significant delay in new secondary tumor development was obtained in the treatment group (see Fig. 1B). Upon longer-term monitoring, however, it became evident that the initial tumor suppression was temporary as all treated tumors resumed growth after week 4. Moreover, even the tumors that had regressed completely, recurred within 2–3 weeks of their eradication, confirming the transient nature of anti-tumor activity in the FVBneuN model.

Earlier studies in transplantable tumor models demonstrated a critical role for T cells in the suppression of established primary tumors following IL-12 + GM-CSF microsphere therapy. To determine whether tumor suppression was mediated by a similar mechanism in the spontaneous tumor setting, in vivo lymphocyte subset depletion studies were performed. $CD4^+$ or $CD8^+$ T cells were depleted by antibody administration

starting 1 day prior to treatment, and the growth of primary tumors was monitored. The results are shown in Figure 1C. Depletion of either $CD4^+$ or $CD8^+$ T-cell subsets resulted in a partial loss of tumor suppression, demonstrating that both subsets were involved in the anti-tumor immune activity. Co-depletion of $CD4^+$ and $CD8^+$ T cells resulted in a near complete loss of tumor suppression, confirming the critical role of T-cell immunity during the initial phase of the anti-tumor response.

Further examination of posttherapy anti-tumor T-cell activity was performed by immunohistochemical analysis of tumor sections obtained from mice at different timepoints after treatment. The goal of this study was to determine whether treatment resulted in a significant infiltration of the tumors by T cells and also to analyze the T-cell infiltration kinetics. To this end, tumor samples were prepared from treated and control mice 1, 3, 7, and 14 days after microsphere injections and were stained with an anti-CD3 antibody. The results are shown in Figure 2. These data demonstrate that T-cell infiltration into tumors began as early as 3 days after treatment, peaked on day 7, and started to diminish by day 14. Analysis of tumor samples at 21 and 28 days post treatment showed a persistent but much reduced level of T-cell infiltration, which became highly variable after week 4 (data not shown). These data establish that whereas treatment induced effective T-cell infiltration into tumors, this activity was transient and correlated with the overall pattern of primary tumor suppression (see Fig. 1A).



Initial Effector T-Cell Activity Is Succeeded by Development of Long-Term Anti-Tumor CTL and Antibody Responses

Suppression of secondary tumor development after IL-12 + GM-CSF microsphere therapy strongly suggested the induction of a long-term, systemic anti-tumor response in addition to the initial transient effector T-cell activity. To determine whether our therapy also induced long-term anti-tumor T-cell memory, splenic T cells obtained from control

FIGURE 1. Primary and secondary tumor growth and the role of T cells in tumor suppression after treatment with IL-12 + GM-CSF microspheres. A, Primary tumor growth. Tumor-bearing mice (100–200 mm^3) were treated with a single intratumoral injection of IL-12 + GM-CSF microspheres, and tumor growth was monitored by weekly measurements of tumor volume. Control mice were treated with blank microspheres (n = 18 and 15 for control and treatment groups, respectively). Error bars = SD. The stars indicate complete tumor regression in 2 of 15 mice in the IL-12 + GM-CSF group. The differences between the two groups were statistically significant on days 7 through 28 ($P < 0.01$, Student t test). B, Secondary tumor development. Development of secondary independent tumors was monitored in control and treated mice. Mice were scored as tumor bearing when the tumor reached 3 mm in diameter. The difference between the groups was statistically significant ($P < 0.04$, log-rank test). C, The role of T-lymphocyte subsets in primary tumor suppression. T-Cell subsets were depleted by in vivo antibody administration starting 1 day prior to treatment as described in Materials and Methods. Tumor growth was monitored weekly. Fold increase in tumor volume for each individual was determined at week 4 post therapy and average fold increase in tumor volume was calculated (n = 9, 9, 5, 5, and 5 for control, no depletion, anti-CD4, anti CD8, and anti-$CD4^+$8 groups, respectively). Error bars = SD. Control = no microsphere treatment and no depletion. All other groups received IL-12 + GM-CSF microspheres and the indicated antibodies (no depletion group received a nonspecific antibody). The difference between control and IL-12 + GM-CSF/no depletion group was significant ($P < 0.005$). The difference between the IL-12 + GM-CSF/no depletion and the anti-CD4 + CD8 groups was also significant ($P < 0.026$). The differences between IL-12 + GM-CSF/no depletion and anti-CD4 or anti-CD8 groups were not significant ($P < 0.131$ and 0.073, respectively).

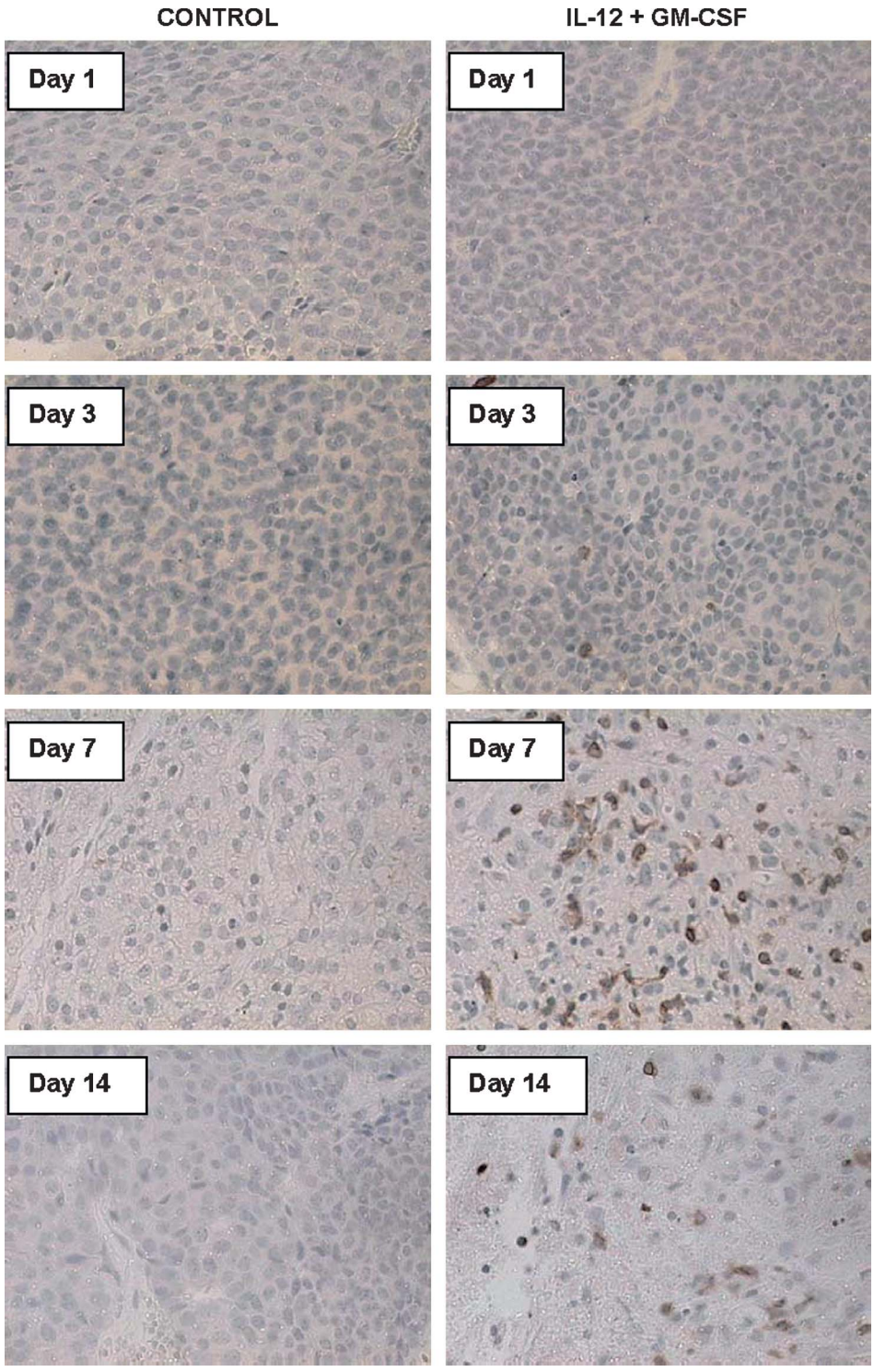


FIGURE 2. Immunohistochemical analysis of posttherapy tumors for T-cell infiltration. Tumor-bearing mice were treated either with control or IL-12 + GM-CSF microspheres, and tumors were removed on indicated days (post treatment) for immunohistochemical staining of tissue sections for $CD3^+$ cells as described in Materials and Methods. Representative images are shown (n = 3 mice/group). Magnification = 400×.

and treated mice 3 weeks after treatment were tested for their ability to kill autologous tumor cell targets. The results are shown in Figure 3A. These data demonstrate that in 7 of 7 mice tested, IL-12 + GM-CSF microsphere treatment resulted in the development of CTLs, although the efficacy of response was variable from mouse to mouse (5%–90% killing at 10:1 effector/target ratio). In mice that were treated with control microspheres, six of seven showed no CTL activity, whereas one mouse displayed an endogenous anti-tumor response. In vitro antibody blocking of major histocompatibility complex

class I presentation abrogated >90% of tumor killing in the above studies, demonstrating that the observed cytotoxicity was mediated by $CD8^+$ cytotoxic T cells (data not shown).

Several studies have suggested that anti-her-2/neu antibody responses play an important role in the suppression of tumors in FVBneuN mice.[18,24] Because both IL-12 and GM-CSF have been shown to be potent adjuvants for B-cell immunity,[25,26] we also investigated whether our strategy induced systemic anti-tumor antibody responses in addition to CTL activation. Mice that were treated either with control or IL-12 + GM-CSF microspheres were bled 18 days after treatment, and the sera were analyzed for the presence of anti-tumor antibodies. The results are shown in Figure 3B. Whereas low levels of anti-tumor antibodies were detected in control mice, significantly higher levels of tumor-specific antibodies were present in the sera of experimental mice, demonstrating that our strategy also enhanced humoral immunity. Furthermore, the anti-tumor antibodies that were present in the sera of treated mice were able to mediate complement deposition on tumor cells, suggesting a possible role for antibody-mediated tumor killing in our model.

To determine whether the systemic anti-tumor immunity could achieve long-term suppression of tumor growth in vivo, splenocytes were isolated from mice treated either with IL-12 + GM-CSF or control microspheres and were transferred adoptively to tumor-free naive recipients 3 weeks after treatment. The recipients were then monitored for tumor development for up to 25 weeks post adoptive transfer. The results shown in Figure 3C establish that splenocytes isolated from immunized donors induced a highly significant, long-term delay of tumor development in naive recipients. Whereas 50% of the mice in the control group developed tumors by week 4 post adoptive transfer, it took 14 weeks for the experimental mice to reach this milestone. Moreover, at week 20 post transfer, 28% of the experimental mice remained tumor-free versus only 4% of the control mice. These experimental mice were still tumor-free 25 weeks post transfer, when the experiment was terminated. These data confirm that a single treatment of the primary tumor results in the development of a potent, long-term systemic anti-tumor response capable of preventing the development of tumors in naive mice upon adoptive transfer. However, this long-term response was not sufficient to maintain the suppression of advanced tumors (see Fig. 1A)

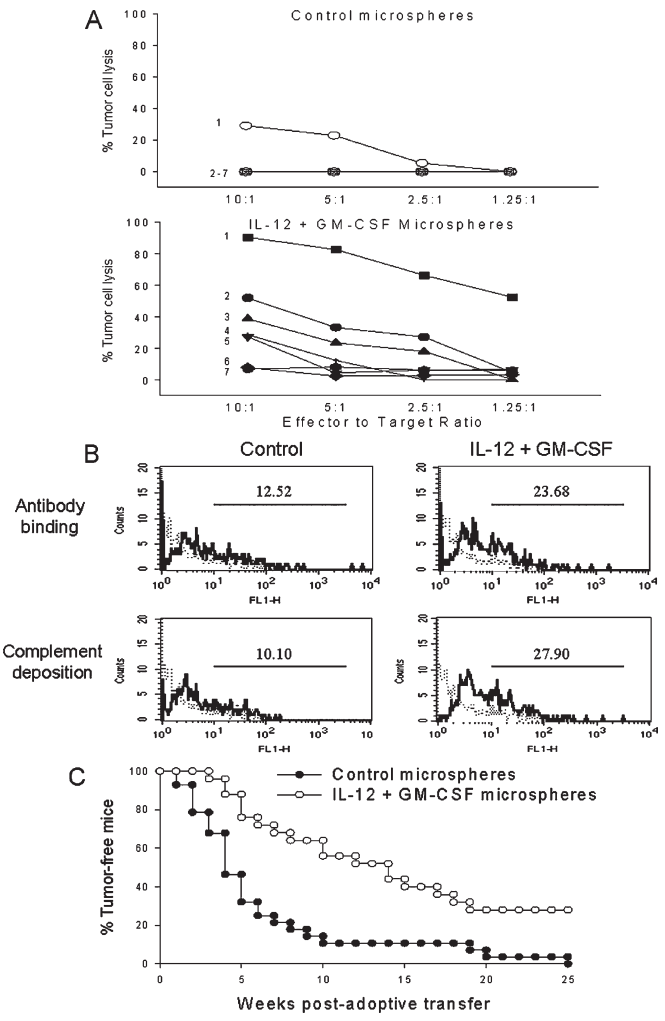


FIGURE 3. Development of anti-tumor CTL and antibodies after IL-12 + GM-CSF microsphere treatment and prevention of tumor development in naive mice following adoptive transfer of splenocytes from immunized mice. A, CTL assay. T cells were prepared from the splenocytes of control and IL-12 + GM-CSF microsphere–treated mice 3 weeks after treatment and were tested for cytotoxicity against autologous tumors (see Materials and Methods). Each line represents a single mouse (n = 7 for both groups). There was no detectable tumor killing in six of seven mice in the control group. B, Anti-tumor antibody development. Mice were treated either with control or IL-12 + GM-CSF microspheres, and serum was collected on day 18 after treatment (n = 3/group). Sera were pooled within each group and tested for the presence of tumor-binding antibodies and their ability to activate complement on tumors by flow cytometry analysis (see Materials and Methods). Dotted line = mouse IgG control; solid line = sera from treated mice. The numbers above the lines indicate the percentage of tumor cells that were positive for antibody binding. C, Adoptive transfer experiment. Tumor-bearing mice were treated either with IL-12 + GM-CSF or control microspheres. Splenocytes were isolated from either group and transferred into age-matched, tumor-free recipients (average age of recipients = 239 ± 45 days). The recipients were then monitored for tumor development twice a week by palpation. Mice were scored as tumor bearing when the tumor size reached 3 mm in diameter. The difference between the control and IL-12 + GM-CSF groups was highly significant ($P < 0.0001$ by log-rank analysis, n = 28 and 23 for control and IL-12 + GM-CSF groups, respectively).

Repeated Treatment of Primary Tumors With IL-12 + GM-CSF Microspheres Enhances Initial Tumor Suppression But Does Not Achieve Complete Cure

Whereas a single treatment was effective in achieving both local and systemic tumor suppression and in some cases the complete regression of established tumors, these effects

were transient, as described above. As T cells were essential to tumor suppression (see Fig. 1C) and the kinetics of intratumoral effector T-cell activity correlated with the transient pattern of tumor suppression (see Figs. 1A and 2), tumor suppression was most likely mediated by the early effector T-cell activity. Thus, we hypothesized that in the case of advanced tumors, the ability to maintain the effector phase may be critical to long-term tumor eradication. To determine whether the intensity of the effector T-cell activity could be maintained by booster treatments, mice were treated with IL-12 + GM-CSF microspheres repeatedly, every 3 weeks. Primary tumor growth, secondary tumor development, and overall survival were then monitored. Results are shown in Figure 4. By week 8 post first treatment, average tumor volume had increased 13-fold in mice that received a single treatment.

A

Number of Treatments	Average fold-increase in primary tumor volume	% Mice with complete primary tumor regression	% Secondary tumor-free mice
Control	N/A	0	31
1	13.1 ± 15.1	13	73
2	2.7 ± 2.6	40	90
3	3.9 ± 2.8	27	73

B

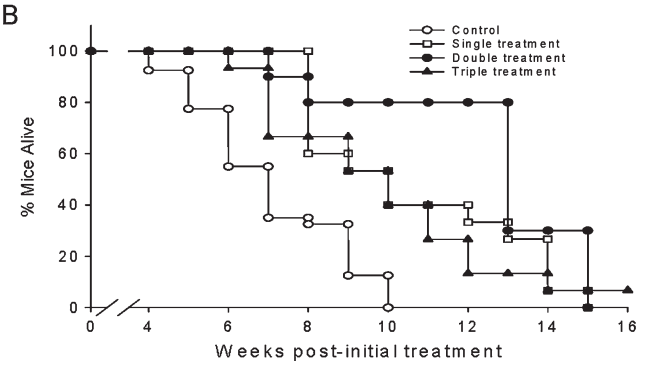


FIGURE 4. Short-term tumor suppression and long-term survival after repeated treatment with IL-12 + GM-CSF microspheres. A, Enhanced short-term suppression of primary and secondary tumors after multiple treatment. Mice with established primary tumors were treated either once, twice, or three times. Treatments were administered every 3 weeks, that is, on weeks 0, 3, and 6 (n = 18, 15, 10, and 13 for control, one, two, and three treatments, respectively). All data shown is for week 8 post first treatment. Fold increase in primary tumor volume is not available for the control group (treated once with blank microspheres) as 10 of 18 control mice were killed before week 8 due to large tumor size (average fold increase in tumor volume at week 4 was 7.1 ± 3.8). The differences between the single-treatment group and double- or triple-treatment groups were significant (P = 0.041 and 0.038, respectively). The difference between double- and triple-treatment groups was not significant (P = 0.32). B, Tumor-bearing mice were monitored for survival after one, two, or three treatments. Mice were killed either when the primary or secondary tumors reached 15 mm in diameter. The differences between control versus single-, double-, or triple-treatment groups were highly significant ($P < 0.0002$) whereas the differences between treatment groups were not significant ($P > 0.1$) as determined by log-rank test (n = 40, 16, 10, and 15 for control, single-, double-, and triple-treatment groups, respectively).

In contrast, tumor size had increased by an average of 3 to 4-fold in mice that received two (weeks 0 and 3) or three (weeks 0, 3 and 6) treatments (see Fig. 4A). A similar pattern was observed with regard to the regression of primary tumors and secondary tumor development by week 8. Complete primary tumor regression was achieved in 13%, 40%, and 27% of mice after one, two, or three treatments, respectively. However, 70% (7/10) of these tumors recurred within 2–4 weeks of their disappearance. Three mice (two in double-treatment and one in triple-treatment groups) remained primary tumor-free for up to 12 weeks but had to be killed owing to the progression of independent secondary tumors. Monitoring of long-term survival demonstrated that a single treatment with IL-12 + GM-CSF microspheres enhanced survival significantly (median survival increased from 7 to 10 weeks and maximum survival increased from 10 to 15 weeks in treated mice; see Fig. 4B). On the other hand, additional treatments did not enhance survival any further. We conclude that chronic immune therapy, though beneficial in the short term, is ineffective in achieving long-term cure of advanced spontaneous tumors in this model.

Failure of Chronic Immunotherapy Is Associated With a Progressive Decline in Intensity of Anti-Tumor T-Cell, But Not of B-Cell, Responses

We next investigated why chronic therapy was ineffective in achieving long-term tumor eradication. To determine whether repeated treatments induced effective immune reactivation in our model, serum IFNγ levels and the degree of T-cell infiltration in posttreatment tumors were analyzed in mice receiving multiple treatments. These data are shown in Figure 5. Serum IFNγ levels, a critical marker for IL-12-induced T- and NK-cell activation,[27] were determined after each treatment. The IFNγ response was highest after the first treatment and declined progressively with increasing number of treatments, suggesting a diminishing T-cell response (see Fig. 5A). Further support for this notion was provided by histologic analysis of tumor samples (see Fig. 5B). Analysis of tumor samples 18 days after the first treatment showed minimal T-cell presence, most likely representing a rapidly contracting T-cell response, as previously demonstrated in Figure 2. A second treatment on day 21 promoted fresh T-cell infiltration (see Fig. 5B, day 28); however, the intensity of secondary infiltration was significantly reduced as compared with day 7 (see Fig. 2). Similarly, diminished T-cell infiltration was observed following the third treatment on day 42 (see Fig. 5B, day 49). These data demonstrate that the intensity of intratumoral effector T-cell activity could not be maintained during chronic therapy.

In contrast to the T-cell data, serum anti-tumor antibody levels increased progressively upon repeated treatment with IL-12 + GM-CSF microspheres (Fig. 6A). This increase was accompanied with a concurrent enhancement of antibody-mediated complement deposition on tumors (see Fig. 6A). Analysis of anti-tumor antibody isotypes demonstrated overall increases in IgG1, IgG2a/b, and IgM subsets (see Fig. 6B),

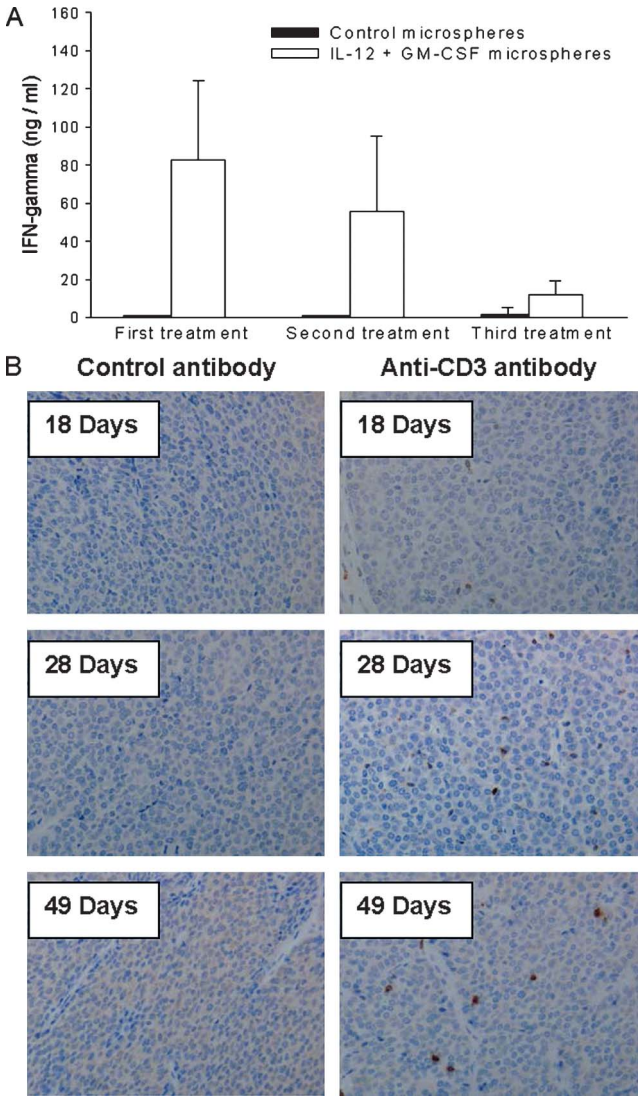


FIGURE 5. Analysis of T-cell activity after repeated treatment with IL-12 + GM-CSF microspheres. A, Posttreatment serum IFNγ levels. Tumor-bearing mice were treated either with control or IL-12 + GM-CSF microspheres (n = 5/group). Sera were collected 3 days after treatment, and IFNγ levels were determined by ELISA. Error bars = SD. The difference between single and double treatments was not significant ($P = 0.207$). The differences between single or double versus triple treatment were significant ($P < 0.008$). B, T-Cell infiltration of tumors. Tumor-bearing mice were treated on days 0, 21, and 42. Tumor sections were obtained 18 days after the first treatment, 1 week after the second treatment (day 28), and 1 week after the third treatment (day 49) and analyzed for CD3-positive cells by immunohistochemistry. Representative images are shown (n = 3/group). Magnification = 400×.

consistent with the co-induction of T_H1- and T_H2-type responses.[28] This is not surprising as IL-12 has been shown to promote IgG2a/b isotype antibodies, whereas GM-CSF primarily induces IgG1 responses.[29] The expansion of the IgM antibodies likely indicates the development of an auto-antigen response with incomplete isotype switching.[30] Whereas these observations establish that repeated treatment enhanced tumor-specific antibody levels, the lack of a correlation between increasing antibody levels and efficacy of long-term tumor suppression does not support a primary anti-tumor role for humoral immunity in these studies.

DISCUSSION

The studies described here establish that in situ tumor vaccination with IL-12 and GM-CSF microspheres induces the regression of advanced spontaneous mammary carcinomas, promotes long-term, tumor-specific T- and B-cell immunity, and enhances long-term survival in the FVBneuN model. To our knowledge, this is the first report where complete regression of advanced spontaneous primary tumors and suppression of multifocal tumor development have been achieved in her-2/neu transgenic mice. These data confirm and extend our previous results obtained in transplantable tumor models[20,21] and provide further proof of principle for the clinical utility of intratumoral cytokine-encapsulated microsphere adjuvants.

Whereas treatment with IL-12 + GM-CSF microspheres was highly effective in the FVBneuN model in the short term, tumor regression was found to be transient upon long-term monitoring. This was in contrast to our earlier results obtained in transplantable tumor models where tumors that regressed upon treatment did not recur.[20,21] The loss of efficacy that accompanies the shift from transplantable to spontaneous tumor models in immunotherapy is well documented[2–7] and is likely due to the fundamental physiologic differences that exist between these models. For example, the gradual progression of spontaneous tumors from premalignant to malignant pathology is accompanied by the development of a growth-promoting and pro-angiogenic tumor stroma[31] as well as the preferential outgrowth of tumor variants with immune-suppressive/evasive phenotypes.[32] Such changes are not commonly observed in rapidly growing transplantable tumors, which potentially make them more vulnerable to immune attack.[6–8] Our results support the notion that spontaneous murine tumor models represent a more challenging and clinically relevant alternative to transplantable tumor models for the preclinical evaluation of tumor immunotherapy protocols.

Analysis of posttherapy anti-tumor immunity was performed to determine the immunologic basis of transient tumor eradication in the FVBneuN model. The results established that tumor regression was primarily mediated by T cells, which homed to and infiltrated into primary tumors within 1 week of treatment. Short-term analysis of T-cell infiltration kinetics demonstrated a correlation between the time courses of intratumoral T-cell activity and tumor regression, suggesting that the early effector phase was critical to tumor eradication. More importantly, however, T-cell infiltration of tumors was found to be transient, with intratumoral T-cell activity contracting within 2–3 weeks of treatment in all mice, independent of tumor regression. The finding that contraction of intratumoral T-cell activity was independent of tumor persistence is not surprising as recent studies have demonstrated the programmed nature of effector T-cell activation kinetics, which was found to be independent of antigen

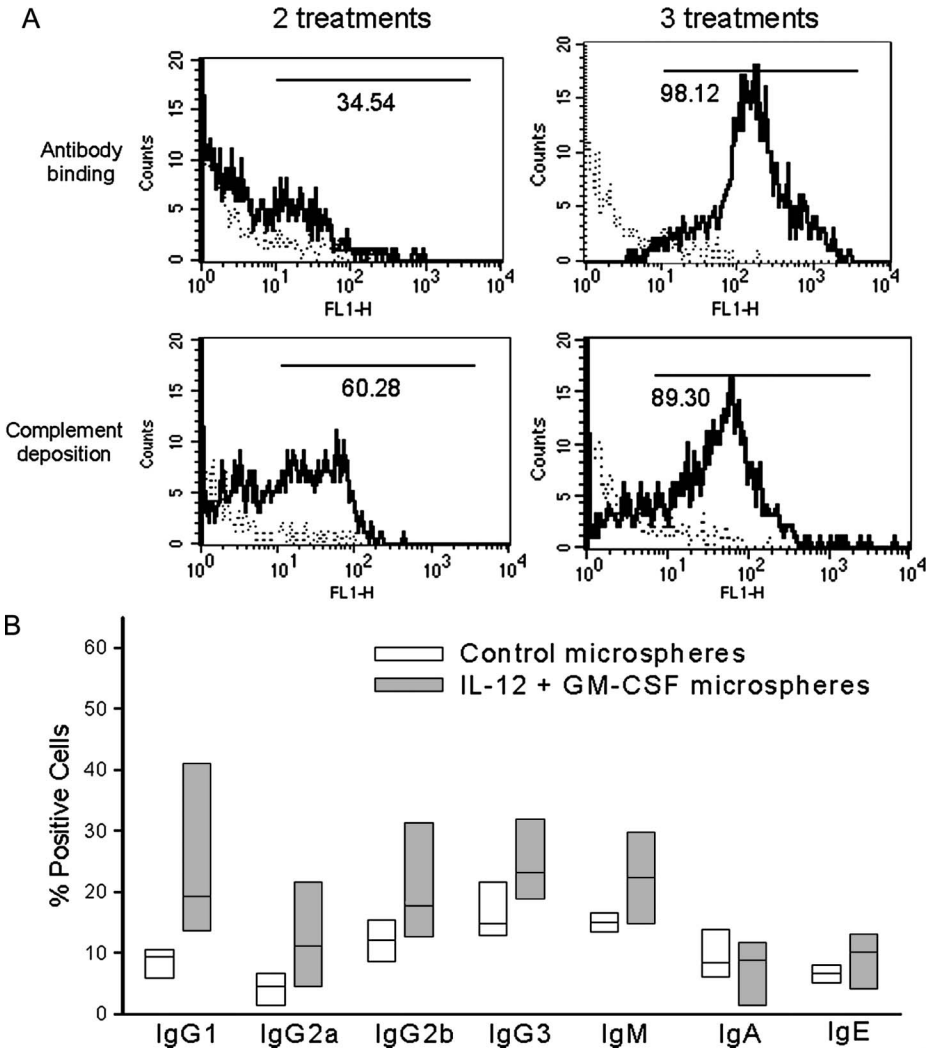


FIGURE 6. Analysis of anti-tumor antibody response after repeated treatment with IL-12 + M-CSF microspheres. A, Serum anti-tumor antibody levels and complement deposition. Tumor-bearing mice were treated either twice (days 0 and 21) or three times (days 0, 21, and 42), and blood was collected 1 week after the second and third treatments. The pooled sera (n = 3/group) were then analyzed for the presence of anti-tumor antibodies and for their ability to mediate complement deposition on tumor cells. The numbers above each line correspond to the percentage of tumor cells positive for antibody binding or complement deposition. B, The pooled serum samples from mice that received three treatments were analyzed for the isotypes of anti-tumor antibodies. The horizontal line within each box plot indicates the median, whereas the vertical length of each box represents the minimum and maximum values for each group (n = 6). The differences between the control and IL-12 + GM-CSF groups were significant for IgG1 and IgM ($P = 0.04$ and 0.05, respectively, Student t test). The differences between IgG2a, IgG2b, IgG3, IgA, and IgE were not statistically significant ($P = 0.07$, 0.09, 0.1, 0.34, and 0.34, respectively).

intensity or persistence.[33] Thus, it is likely that spontaneous FVBneuN tumors outlast this narrow window of effector T-cell activity more successfully than transplantable tumors, explaining, at least in part, the differential ability of our strategy to achieve complete cure of transplantable but not spontaneous tumors in the long term.

In addition, it was also possible that our treatment, although effective in promoting effector T-cell activity, failed to induce a long-term memory response in this model, resulting in posteffector tumor resurgence. Studies designed to address this possibility demonstrated the development of a long-term, systemic anti-tumor immune response with a significant CTL component, which prevented tumor development in naive mice upon adoptive transfer. However, the long-term memory response was still unable to maintain the suppression of established, advanced primary tumors in tumor-bearing individuals, even after the primary tumor was largely eradicated by effector T cells. One possible explanation for the inefficacy of long-term T-cell immunity is the potential development of tumor escape variants.[32] Although this possibility cannot be ruled out here, the rapid recurrence of tumors in our model (within 2 weeks of remission) is not consistent with this notion. An alternative explanation involves the tolerization of anti-tumor T cells by a persisting tumor, which has been demonstrated to occur in both murine and human studies.[34–36] We are currently investigating whether the inability of posteffector T cells to maintain long-term suppression of tumors in FVBneuN mice is due to changes in tumor phenotype and/or loss of T-cell function.

Because the initial effector T-cell activity was found to be critical to tumor eradication in our model, we hypothesized that reactivation of the effector phase by repeated booster treatments with IL-12 + GM-CSF microspheres could lead to long-term tumor eradication. When this approach was tested, a short-term enhancement of tumor suppression was observed, but long-term cure was not achieved. In fact, repeated treatment resulted in a progressive weakening of the intratumoral effector T-cell response after each treatment. In a recent study, repetitive in vitro stimulation of tumor-specific T cells isolated from tumors resulted in the emergence of regulatory $CD4^+$ T cells.[37] The presence of T-suppressor/regulatory cells in murine and human tumors and their ability to suppress

anti-tumor immune responses have been demonstrated.[38,39] Thus, one likely explanation for the progressive loss of effector T-cell activity after repetitive treatment is a concurrent expansion of the regulatory T-cell component in our model. If this is the case, strategies designed to counteract regulatory mechanisms, such as CTLA-4 blockade or depletion of T-regulatory cells, which have been shown to dramatically improve the efficacy of T-cell-based vaccines, could potentially enhance the efficacy of the approach described here.[1,2]

The above observations have important implications for the long-term clinical utility of the particular strategy described here (and potentially vaccine-based therapies in general) in patients with advanced disease. The transient nature of the intratumoral effector T-cell activity and the inability to resurrect this response in the FVBneuN mice suggest that treatment with IL-12 + GM-CSF microspheres alone may have limited curative potential in the established disease setting. Strategies that target immune-regulatory/homeostatic mechanisms in combination with standard vaccination have shown superior efficacy in comparison with vaccination alone.[1,2] Whether the use of such combinatory approaches in concert with IL-12 + GM-CSF microsphere therapy can lead to complete cure of advanced tumors in the FVBneuN model remains to be determined.

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Chronic Immune Therapy Induces a Progressive Increase in Intratumoral T Suppressor Activity and a Concurrent Loss of Tumor-Specific CD8$^+$ T Effectors in her-2/neu Transgenic Mice Bearing Advanced Spontaneous Tumors[1]

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A single intratumoral injection of IL-12 and GM-CSF-encapsulated microspheres induces the complete regression of advanced spontaneous tumors in her-2/neu transgenic mice. However, tumor regression in this model is transient and long-term cure is not achieved due to recurrence. Posttherapy molecular analysis of immune activation/suppression markers within the tumor microenvironment demonstrated a dramatic up-regulation of IFN-γ and a concomitant down-regulation of Forkhead/winged-helix protein 3 (Foxp3), TGFβ, and IL-10 expression. Therapy-induced reversion of immune suppression was transient since all three markers of suppression recovered rapidly and surpassed pretherapy levels by day 7 after treatment, resulting in tumor resurgence. Repeated treatment enhanced short-term tumor regression, but did not augment long-term survival. Serial long-term analysis demonstrated that although chronic stimulation enhanced the IFN-γ response, this was countered by a parallel increase in Foxp3, TGFβ, and IL-10 expression. Analysis of tumor-infiltrating T lymphocyte populations showed that the expression of Foxp3 and IL-10 was associated with CD4$^+$CD25$^+$ T cells. Repeated treatment resulted in a progressive increase in tumor-infiltrating CD4$^+$CD25$^+$Foxp3$^+$ T suppressor cells establishing their role in long-term neutralization of antitumor activity. Analysis of tumor-infiltrating CD8$^+$ T cells demonstrated that although treatment enhanced IFN-γ production, antitumor cytotoxicity was diminished. Monitoring of CD8$^+$ T cells that specifically recognized a dominant MHC class I her-2/neu peptide showed a dramatic increase in tetramer-specific CD8$^+$ T cells after the first treatment; however, continuous therapy resulted in the loss of this population. These results demonstrate that both enhanced suppressor activity and deletion of tumor-specific T cells are responsible for the progressive loss of efficacy that is associated with chronic immune therapy. *The Journal of Immunology,* **2006, 176: 7325–7334.**

Tumor vaccines can successfully promote the development of tumor-specific cytotoxic T cell responses in both murine tumor models and cancer patients (1, 2). In contrast, induction of antitumor T cells rarely results in clinical tumor regression (3, 4). Postvaccine antitumor T cell immunity is routinely monitored by quantification of tumor-specific T cells in peripheral blood (5). However, peripheral T cell activity is a poor correlate for intratumoral T effector cell function (2, 4, 6), because tumors can actively subvert immune responses by producing immune inhibitory molecules and by attracting and retaining T suppressor cells (7). Therefore, combinatorial approaches that are designed to concurrently prime tumor-specific T cell responses and to neutralize the immune suppressive elements in the tumor microenvironment are more likely to achieve effective tumor regression.

The cytokine milieu of the tumor microenvironment is critical to the outcome of the interaction between the tumor and the immune system (7, 8). Modulation of the tumor microenvironment by local delivery of proinflammatory cytokines can overcome tumor immune suppression and induce both local and systemic antitumor regression (8). To this end, we demonstrated that a single intratumoral injection of IL-12 and GM-CSF-encapsulated biodegradable, sustained-release microspheres can achieve the complete regression of established primary tumors, induce long-term systemic antitumor T cell immunity, and eradicate established metastatic disease in a transplantable murine lung tumor model (9, 10). More recently, we evaluated (11) the curative potential of this in situ vaccination approach in her-2/neu transgenic mice, who spontaneously develop mammary tumors. These studies demonstrated that in situ delivery of IL-12 and GM-CSF achieved complete regression of advanced spontaneous mammary tumors and promoted the development of long-term, protective T and B cell responses. In contrast to the results obtained in the transplantable tumor model, tumor eradication was found to be transient in this model because most lesions eventually recurred. Repeated treatment of recurring tumors improved therapeutic efficacy in the short term; however, long-term survival was not enhanced beyond what was achieved with a single treatment due to recurrence. Preliminary analysis of tumors in mice receiving repeated treatment demonstrated a progressive decline in the intensity of posttherapy T cell activity within the tumor microenvironment (11).

Recently, it was reported (12) that repeated vaccination promotes a progressive quantitative increase in the frequency of peripheral antitumor T cells, but that this increase does not result in enhanced tumor regression in patients. Functional integrity of vaccine-induced, tumor-infiltrating T cells was not monitored in this study. Because chronic stimulation failed to maintain intratumoral T cell activity in our studies, we hypothesized that the observed lack of correlation between enhanced peripheral T effector activity

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and tumor regression could be due to a concurrent augmentation of T suppressor cell activity in the tumor microenvironment. To this end, quantitative monitoring of T effector cell activity and the accompanying T suppressor cell responses in tumors has not been performed during chronic therapy. To determine whether repeated treatment of advanced spontaneous mammary tumors in her-2/neu transgenic mice resulted in such a response-counterresponse process, we monitored the quantitative and qualitative changes in immune activation and suppression markers in posttherapy tumors. The results establish that intratumoral delivery of IL-12 and GM-CSF promotes a dramatic reversal of immune suppression in the tumor microenvironment but that this reversion is transient because intratumoral T suppressor cell activity recovers rapidly. Importantly, our data demonstrate that whereas repeated treatment resurrects antitumor immune activity, reactivation is countered by a concurrent and progressive increase in the intensity of T suppressor cell infiltration into tumors, resulting in reduced T effector cytotoxicity and eventual deletion of tumor-specific CTL.

Materials and Methods

Mice

Five breeder pairs of FVB/N or FVB/N-TgN $^{MMTV\text{neu}}$202Mul (FVBneuN) at 6–8 wk of age were purchased from The Jackson Laboratory. Mice were bred and female progeny were maintained in microisolation cages (Laboratory Products) in the barrier unit of the University of Louisville's Laboratory Animal Facility. All other strains were purchased from The Jackson Laboratory. Tumor development was monitored by palpation once a week after mice reached 4 mo of age. All studies were approved by the Institutional Animal Care and Use Committee of the University of Louisville.

Cytokines and microspheres

Recombinant murine IL-12 (2.7×10^6 U/mg) was a gift from Wyeth-Averst Pharmaceuticals. Recombinant murine GM-CSF (5×10^6 U/mg) was purchased from PeproTech. Preparation of cytokine-encapsulated biodegradable polymer microspheres was described in detail previously (11).

Microsphere treatments, monitoring of tumor growth, and survival

Mice were monitored for tumor development by palpation twice a week. All mice developed tumors between 175 and 330 days of age. Treatment was administered with a single intratumoral injection of microspheres when tumors reached ~100–200 mm^3 in size (tumor volume was determined according to the formula $A \times B^2/2$, where A is the longest and B is the shorter perpendicular dimension of the tumor). Experimental groups received IL-12 plus GM-CSF-encapsulated microspheres (bioactivity equivalent of 2 μg cytokine) suspended in 150 μl of hydration buffer (1% hydroxypropylmethylcellulose (Dow) and 1% Pluronic F-127 (Sigma-Aldrich) in PBS, pH 7.2). Control mice received blank microspheres in 150 μl of hydration buffer. Mice were sacrificed when the largest diameter of the tumor reached 15 mm.

Quantitative real-time PCR analysis

Fine needle aspirates were obtained by aspirating four quadrants of each tumor with a 23¾-gauge needle attached to a 1.0-ml syringe. Tissue samples were discharged into TRIzol (Invitrogen Life Technologies); total mRNA was isolated and was reverse-transcribed with TaqMan Reverse Transcription reagents (Applied Biosystems). IFN-γ, IL-10, Forkhead/winged-helix protein 3 (Foxp3)[3], TGF-β, indoleamine 2,3 dioxygenase (IDO), and GAPDH mRNA levels were quantified by real-time RT-PCR amplification using the Mx3000PTM Real-Time PCR System (Stratagene) as recommended by the manufacturer. Briefly, target transcripts were amplified in a 25-μl reaction mixture containing 12.5 μl of SYBR Green PCR Master Mix (Applied Biosystems), 100 ng of cDNA template, and selected primers (200 nM) using the recommended cycling conditions (denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). The primer sequences, designed with Primer Express software (Applied Biosystems), were as follows: Foxp3, 5′-TCCCACGCTCGGGTACAC-3′ (forward) and 5′-TTGCCAGCAGTGGGTAGGAT-3′ (reverse); IFN-γ, 5′- GCACAGTCATTGAAAGC-3′ (forward) and 5′- TGCCAGTTCCTCCAGATA-3′ (reverse); IL-10, 5′-CCTGGTAGAAGTGATGCCCC-3′ (forward) and 5′-TCCTTGATTTCTGGGCCATG-3′ (reverse); GAPDH, 5′-TCCTTGATTTCTGGGCCATG-3′ (forward) and 5′-TCTTCTGGGTGGCAGTGATG-3′ (reverse); TGF-β, 5′-CGCTTGCAAAACCCCAAA-3′ (forward) and 5′-TTTCCATTTAAGGATCTGA TACAGTTCA-3′ (reverse); and IDO, 5′-CAGGCCAGAGCAGCATCTTC-3′ (forward) and 5′-GCCAGCCTCGTGTTTTATTCC-3′ (reverse). Relative quantification of mRNA expression was calculated by the comparative cycle threshold (Ct) method (13). The relative target quantity, normalized to an endogenous control (GAPDH) and relative to the day 0 calibrator, was expressed as $2^{-\Delta\Delta Ct}$ (fold), where ΔCt = Ct of the target gene (IFN-γ, Foxp3, IL-10, TGF-β) − Ct of endogenous control gene (GAPDH), and $\Delta\Delta Ct = \Delta Ct$ of samples for target gene − ΔCt of the 0-day calibrator for the target gene.

Preparation of single-cell suspensions from tumors

Tumors were disaggregated by a modification of the enzyme digestion technique originally described by Russell et al. (14). Briefly, tumors were excised and minced with sterile scissors and a surgical blade into ~1–2 mm^3 pieces. The minced tumor fragments were then digested in DMEM/F12 containing 10% FCS and 0.05 mg/ml collagenase D (Roche Molecular Systems), 0.02 mg/ml hyaluronidase type V (Sigma-Aldrich), and 0.01 mg/ml DNase I (Sigma-Aldrich), in PBS plus 0.5% BSA at 37°C in a rotating platform for 1 h. The supernatant was filtered through 70-μm nylon mesh (Falcon 2340; BD Biosciences) to remove clumps and washed immediately in cold DMEM/F12. The cell suspension containing $5–10 \times 10^7$ viable cells in 5 ml of DMEM/F12 was layered over 3 ml of 20% Ficoll (Amersham Biosciences) solution in a 15-ml conical bottom centrifuge tube and centrifuged for 20 min at $1350 \times g$. Live cells (tumor cells and tumor-infiltrating leukocytes (TIL)) were collected from the interface and analyzed by flow cytometry.

Flow cytometry

Cells were stained using standard techniques (15) and analyzed on a four-color BD FACSCalibur flow cytometer (BD Biosciences). The following mAbs were used: FITC-conjugated anti-mouse CD4 (GK1.5), FITC- conjugated anti-mouse CD8 (53-6.7), and allophycocyanin-conjugated anti-mouse CD25 (PC61). Background levels were determined with isotype-matched control Abs. All Abs were purchased from BD Pharmingen.

Intracellular cytokine staining

Intracellular staining for IFN-γ and IL-10 was performed using the BD Pharmingen kit for detection of IFN-γ and IL-10 as recommended by the manufacturer (BD Pharmingen). Briefly, 1×10^6 cells were incubated in CTL medium (RPMI 1640 plus 10% FBS, 0.5% L-glutamine, 10 μg/ml penicillin/streptomycin, and 50 μM 2-ME, pH 7.1) containing PMA (50 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiStop. Cells were washed in FACS buffer (BD Pharmingen), stained with FITC-conjugated anti-mouse CD8 (53-6.7), fixed, permeabilized, and stained with PE-conjugated anti-mouse IFN-γ (XMG1.2). For detection of intracellular IL-10, cells were stained with FITC-conjugated anti-mouse CD4 (GK1.5) and allophycocyanin-conjugated anti-mouse CD25 (PC61), fixed, permeabilized, and stained with PE-conjugated anti-mouse IL-10 (JES5-16E3). Intracellular staining for Foxp3 was performed using a Foxp3 staining kit (eBioscience). Cells were stained with FITC-conjugated anti-mouse CD4(GK1.5) and allophycocyanin-conjugated anti-CD25 (PC61), fixed, permeabilized, and stained with PE-conjugated anti-mouse Foxp3 (FJK-16s; eBioscience). All Abs were from BD Pharmingen unless indicated otherwise.

IFN-γ ELISPOT assay

Assays were performed using the Mouse IFN-γ ELISPOT plus kit (Mabtech). Briefly, 96-well ELIIP10SSP polyvinylidene fluoride plates (Millipore) were coated at 4°C overnight with 3 μg/ml capture Ab (anti-IFN-γ Ab AN-18; Mabtech). The plates were then washed and blocked with DMEM/F12 with 10% FCS for 1 h at 37°C. TIL or tumor-draining lymph nodes (TDLN) cells (2×10^5/well) were cultured in 100 μl of culture medium either with anti-mouse CD3 (1μg/ml; BD Pharmingen) plus IL-2 (5 ng/ml) or RNEU$_{420-429}$ peptide PDSLRDLSVF (16) for 48 h at 37°C and 5% CO_2 in duplicate wells with 1/2 serial dilutions. After culture, the plates were washed and incubated first with 1 μg/ml biotinylated anti-IFN-γ Ab (Mabtech), then with 1/1000 streptavidin-alkaline phosphatase (Mabtech), and finally with 5-bromo-4-chloro-3-indolyl phosphate/NBT; Mabtech). The plates were developed at room temperature for 20–30 min until visible spots appeared. The reaction was then stopped by washing extensively in tap water. The plates were air-dried, and the spots

[3] Abbreviations used in this paper: Foxp3, Forkhead/winged-helix protein 3; TIL, tumor-infiltrating leukocyte; TDLN, tumor-draining lymph node; IDO, indoleamine 2,3-dioxygenase; FNA, fine needle aspiration.

were counted with a dissecting microscope. The frequency of cytokine-producing cells was expressed as the difference between the mean number of spots after stimulation and the mean background without stimulation. The background was 9.4 ± 2.3 spots/well.

Cytotoxicity assays

Mice were euthanized by CO_2 inhalation, TDLN were removed and mechanically disaggregated through a 70-μm cell strainer. The cell suspension was then enriched for $CD8^+$ T cells using the SpinSep-negative selection separation system according to the manufacturer's instructions (StemCell Technologies). The purity of the $CD8^+$ T effector cell preparation was >95% as determined by flow cytometry. Cell-mediated cytotoxicity was measured by a dye exclusion assay (17, 18). Target cells were prepared and pooled from established tumors of six to eight mice as described previously (11). After washing once in PBS, tumor cells were stained with PKH26 using the PKH26-GL dye kit (Sigma-Aldrich) as recommended by the manufacturer. PKH26-labeled target cells were then suspended at $2–4 \times 10^5$ cells/ml in DMEM/F12 and 100-μl aliquots were added to FACS tubes for the CTL assay. $CD8^+$ T effectors (100 μl) at various concentrations were mixed with target cells, tubes were centrifuged at $200 \times g$ for 2 min, and incubated at 37°C for 48 h. Following incubation, 1 μl of 1 mg/ml stock solution of propidium iodide was added to each tube and the tubes were incubated for 15 min at 37°C before analysis by flow cytometry. Five thousand PKH26-labeled target cells were acquired per sample. The extent of cytotoxicity was determined by quantification of dead cells labeled with both PKH-26 plus propidium iodide and live cells labeled with PKH-26. Cytotoxicity was reported as percentage of cell death within the PKH-26 plus targets ((dead-labeled targets/dead-labeled targets plus live-labeled targets) multiplied by 100). Percentage target cell death was corrected for spontaneous background death by subtracting the percentage of dead cells in control samples (PKH-26-labeled targets alone) from the percentage of dead cells within the test samples.

Tetramer analysis

Recombinant allophycocyanin-conjugated H-2Dq tetramers bound to neu-specific peptide PDSLRDLSVF were produced by the MHC Tetramer Core Facility at the National Institute of Allergy and Infectious Diseases (Bethesda, MD). The TIL (prepared as single-cell suspensions from tumors as described above) and TDLN cells were stained with H-2Dq tetramer for 30 min on ice and surface stained with FITC-conjugated anti-mouse CD8 (53-6.7). Cells were then fixed and permeabilized with a Cytofix/Cytoperm kit (BD Pharmingen), followed by intracellular staining for IFN-γ as described above.

Results

A single injection of IL-12 plus GM-CSF microspheres results in a dramatic reversal of intratumoral immune suppression and promotes enhanced survival

To determine how repeated treatment with IL-12 and GM-CSF-loaded microspheres affected tumor-infiltrating T lymphocyte activity, we monitored the expression of intratumoral T cell activation (IFN-γ) and suppression (Foxp3, TGFβ, and IL-10) markers by quantitative real-time PCR. Initially, we analyzed the effect of

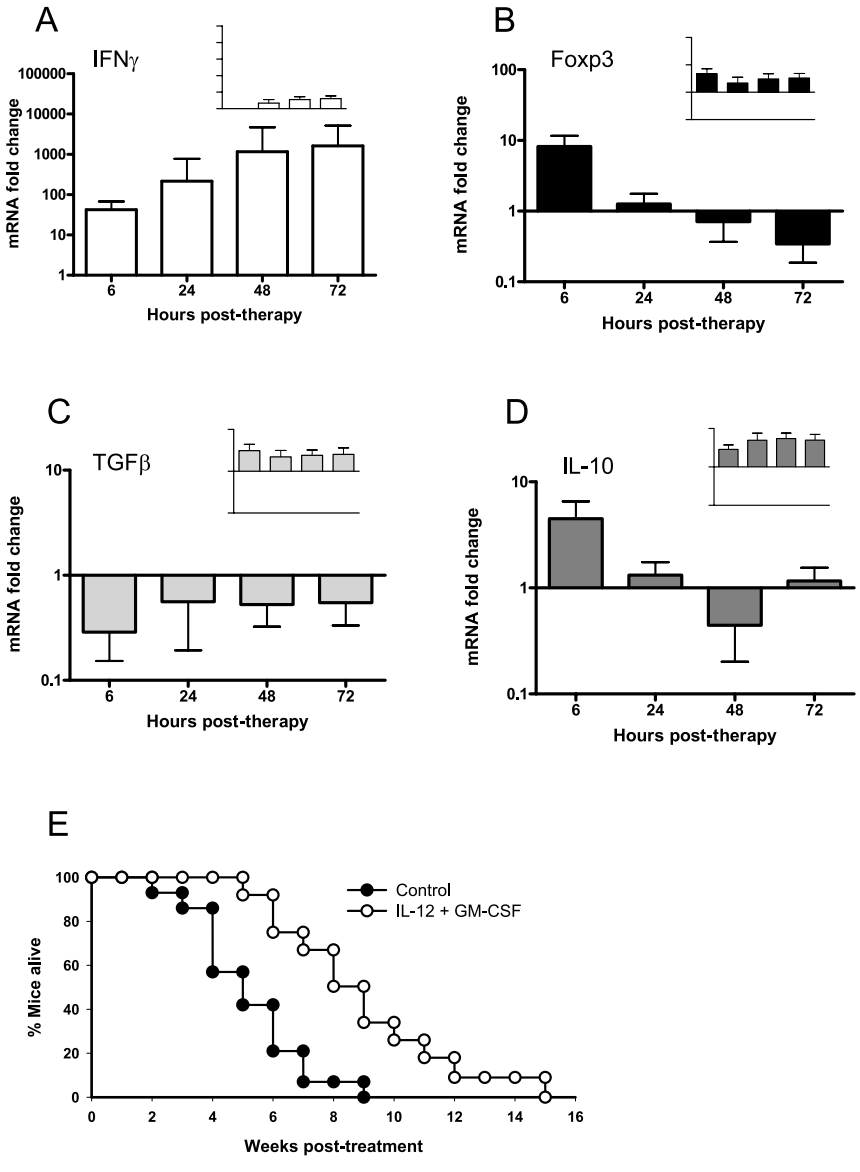

FIGURE 1. Effect of IL-12 + GM-CSF microsphere treatment on intratumoral IFN-γ, Foxp3, TGFβ, and IL-10 expression. FNA samples were obtained from tumors before treatment and at indicated times after treatment. Total RNA was analyzed for the expression of selected markers by real-time PCR as described in *Materials and Methods*. The fold change is relative to pretreatment levels of each transcript (1-fold). *A–D*, Samples were obtained from six to eight mice per time point. Error bars, SD. *Inset*s, The results from mice treated with control (blank) microspheres. *E*, Survival of mice after a single treatment. Control mice received an injection of blank microspheres. The difference between the survival of control and IL-12 plus GM-CSF groups was highly significant as determined by log-rank analysis ($p = 0.0007$, $n = 14$ and $n = 12$ for control and IL-12 plus GM-CSF, respectively).

therapy on the selected markers in short-term studies to demonstrate that treatment altered their expression profiles, thus validating the rationale for this approach. Mice bearing spontaneous advanced (100–200 mm^3) mammary tumors were treated either with a single intratumoral injection of IL-12 plus GM-CSF-encapsulated microspheres or control (blank) microspheres. Tissue samples were obtained from tumors by fine needle aspiration (FNA) before therapy and at different time points following treatment (19), and total RNA was analyzed for IFN-γ, Foxp3, TGFβ, and IL-10 transcript levels by real-time PCR. The results are shown in Fig. 1. These data demonstrate that treatment promoted a rapid and dramatic increase in intratumoral IFN-γ transcript levels as early as 6 h posttherapy, which continued to increase until day 3 (Fig. 1*A*). More importantly, the dramatic up-regulation of IFN-γ during the first 3 days was accompanied by a concomitant decrease (up to 4-fold) in markers of immune suppression, i.e., Foxp3, TGFβ, and IL-10 (Fig. 1, *B–D*), demonstrating an effective reversal of the immune suppressive characteristics of the tumor microenvironment. In contrast, control-treated mice neither showed a significant increase in IFN-γ (Fig. 1*A*, *inset*) nor a decrease in any of the suppression markers (Fig. 1, *B–D*, *insets*). Treatment-induced reversal of immune suppression resulted in improved long-term survival in treated mice as compared with control-treated animals (Fig. 1*E*).

Treatment-induced reversal of intratumoral immune suppression is transient and repeated treatment results in progressively higher rebound levels of Foxp3, TGFβ, and IL-10 in tumors

The above results demonstrated that IL-12 plus GM-CSF microsphere treatment resulted in a dramatic conversion of the immune-suppressive tumor milieu to one that is immunologically active, at least in the short term. To determine whether this reversion persisted, posttherapy intratumoral IFN-γ, Foxp3, TGFβ, and IL-10 levels were monitored further, initially up to 20 days after the first treatment, and then for up to 7 wk during repeated therapy. The long-term real-time PCR data for the four selected markers are shown in Fig. 2. These results again demonstrate that treatment with IL-12 plus GM-CSF microspheres reversed the immune suppressive character of the tumor microenvironment as indicated by the >30-fold increase in intratumoral levels of IFN-γ and a 2- to >10-fold decrease in the levels of Foxp3, TGFβ, and IL-10 within 2–3 days after treatment. This reversal, however, was transient and transcript levels of all three markers of suppression recovered rapidly. This recovery proceeded despite the continued presence of high quantities of IFN-γ and in fact surpassed pretreatment levels by 10- to 20-fold as early as day 7. By day 20 posttreatment, IFN-γ expression had declined; however, Foxp3, TGFβ, and IL-10 transcript levels remained high and, in the case of IL-10 and TGFβ, continued to increase, reaching >200-fold above pretherapy

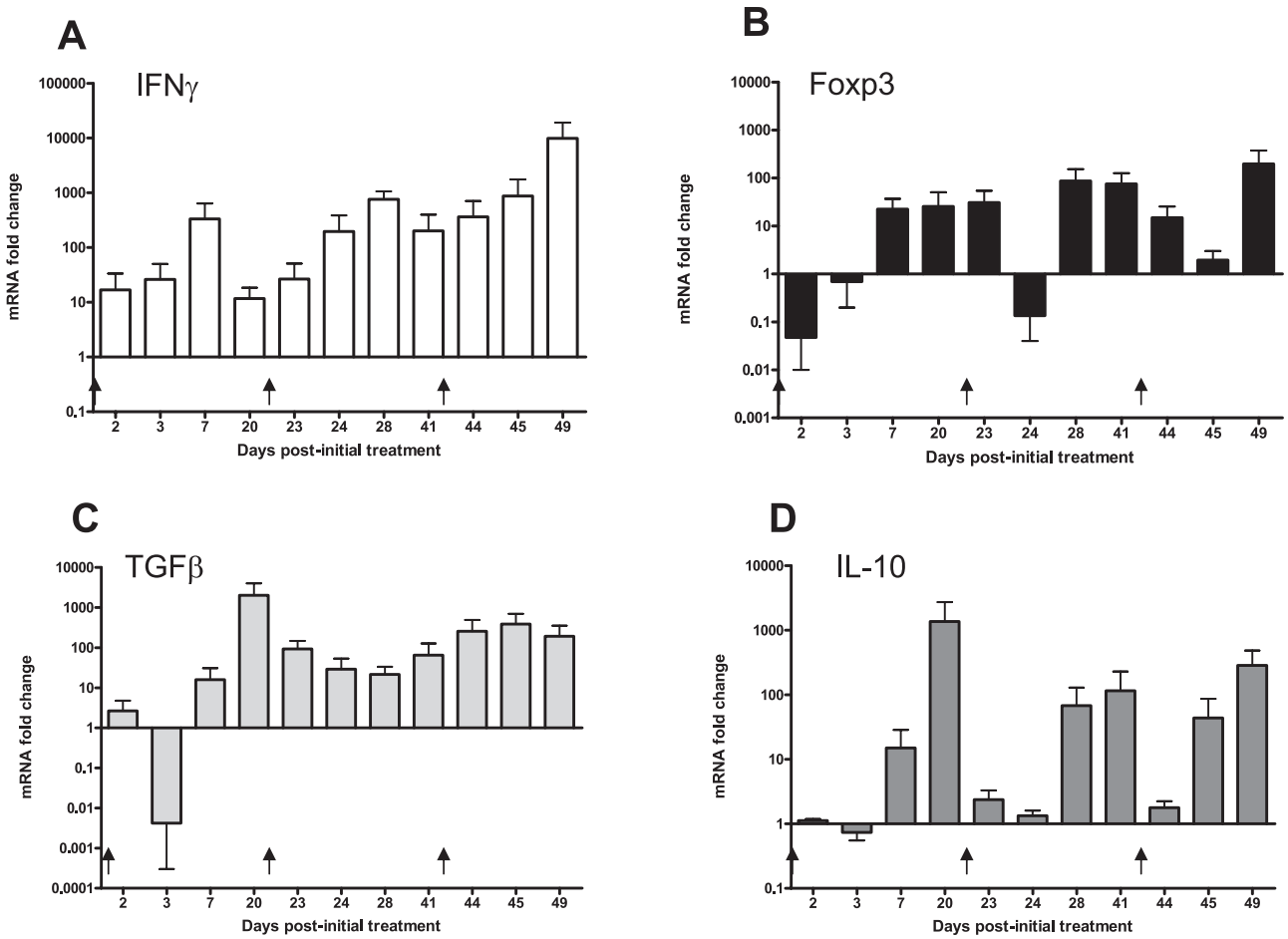

FIGURE 2. Effect of repeated IL-12 plus GM-CSF microsphere treatment on long-term IFN-γ, Foxp3, TGFβ, and IL-10 expression in tumors. FNA samples were obtained from tumors before treatment (day 0) and on indicated days after treatment. Total RNA from each sample was analyzed for the expression of selected markers by real-time PCR as described in *Materials and Methods*. The fold change is relative to day 0 pretreatment levels of each transcript. Values are averages of samples from three to eight mice per time point. FNA samples could not be obtained from one mouse after day 7 due to complete tumor regression and from three other mice at different time points due to transient tumor regression at the time. After day 35, three mice had to be sacrificed due to tumor growth before FNA samples could be procured. Error bars, SD. Arrows indicate time of treatment (days 0, 21, and 42).

levels (Fig. 2). These data establish that treatment-induced reversal of tumor immune suppression was brief and was followed by a rapid recovery of suppression markers to levels that exceeded pretherapy intensity.

To determine whether the T cell activity within the tumor microenvironment could be resurrected, tumors were injected again on days 21 and 42 after the first treatment. Upon administration of the second treatment on day 21, a significant increase in IFN-γ and a concomitant decline in Foxp3, TGFβ, and IL-10 levels were achieved again (Fig. 2). In fact, the second treatment induced much higher levels of IFN-γ than the first treatment (>190-fold increase over pretherapy levels on day 24 vs 30-fold on day 3). Consistent with this increase, the reversal of suppression was highly effective, resulting in >200-, 70-, and 500-fold decreases in Foxp3, TGFβ, and IL-10 transcript levels between days 20 and 24, respectively (Fig. 2, *B–D*). In contrast, analysis of day 28 samples showed a rapid recovery of the suppressive factors between days 24 and 28 similar to that seen after the first treatment, except that the absolute transcript levels were now significantly higher (cf. days 7 and 28). After the third treatment (on day 42), there was again a dramatic increase in IFN-γ levels (>9000-fold higher than pretherapy levels); however, suppression of Foxp3, TGFβ, and IL-10 was less effective than before (cf. changes between days 41 and 45). In fact, TGFβ no longer appeared to respond to treatment. The absolute transcript levels for suppression markers 1 wk after the third treatment (day 49) were higher than the levels reached after the second treatment (>195-, 190-, and 280-fold above pretherapy levels on day 49 vs >85-, 20-, and 65-fold on day 28 for Foxp3, TGFβ, and IL-10, respectively), demonstrating the progressive nature of regulatory recovery with each treatment. These results establish, in a quantitative manner, that although repeated treatment promoted progressively stronger immune activation in the tumor microenvironment as measured by IFN-γ levels, suppressive mechanisms countered this activation with increasing intensity.

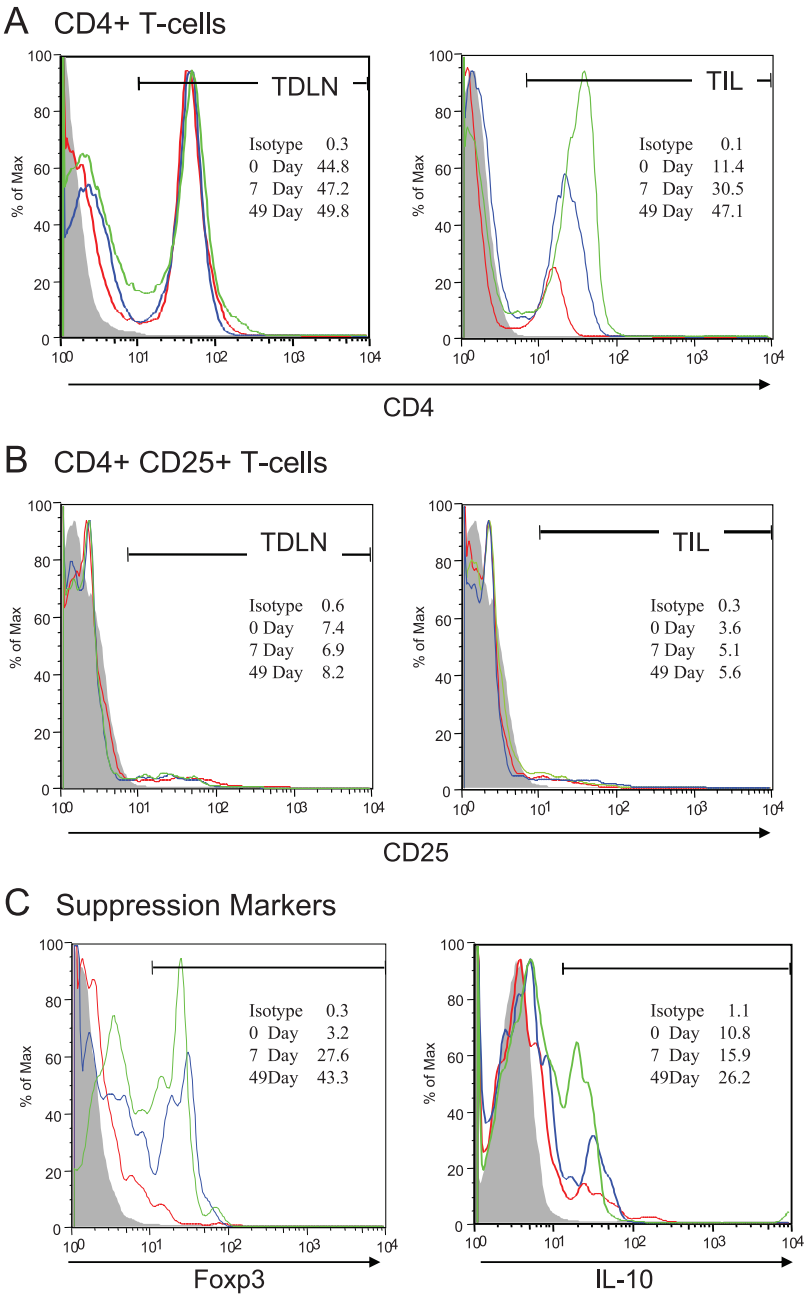

FIGURE 3. Effect of treatment on intratumoral T suppressor cell activity. TDLN cells and single-cell suspensions prepared from tumors were analyzed by four-color flow cytometry as described in *Materials and Methods*. Cells were pooled from three separate mice for each time point. Results are representative of at least two, in some cases three, independent experiments. *A*, Lymphocytes were gated on and analyzed for CD4 expression. *B*, $CD4^+$ cells were gated on and analyzed for CD25 expression. *C*, $CD4^+CD25^+$ T cells were gated on and analyzed for Foxp3 and IL-10 expression by intracellular staining. Gray shading, isotype control; red, day 0; blue, day 7; and green, day 49.

The progressive increase in intratumoral Foxp3 and IL-10 expression that accompanies repeated treatment is associated with enhanced T suppressor cell infiltration

Because Foxp3, TGFβ, and IL-10 are all expressed by $CD4^+CD25^+$ T suppressor cells, we quantitatively monitored $CD4^+CD25^+$ T cells in pre- and posttherapy tumors to determine whether repeated treatment resulted in increased T suppressor infiltration. For this, lymphocyte populations were isolated from tumors as well as TDLN before treatment (day 0), 1 wk after the first treatment (day 7), and 1 wk after the third treatment (day 49), and $CD4^+CD25^+$ T cells were quantified by flow cytometry. The results are shown in Fig. 3. There were no differences in the percentage of $CD4^+$ T cells in the TDLN obtained from 0-, 7-, and 49-day mice (Fig. 3*A*). In contrast, the proportion of the $CD4^+$ T cells within the TIL increased progressively between days 0 and 49 (>4-fold; Fig. 3*A* and Table I). The proportion of the $CD4^+$ $CD25^+$ subset within the $CD4^+$ T cell population did not change in the TDLN but expanded in tumors (1.5- to 3.8-fold in different experiments) between days 0 and 49 (Fig. 3*B*). The overall increase in $CD4^+CD25^+$ cells within the TIL population exceeded 12-fold between days 0 and 49 (Table I). The progressive rise in the $CD4^+CD25^+$ T cell numbers between days 0 and 49 mirrored the real-time PCR data; therefore, suggesting that the increase in Foxp3, TGFβ, and IL-10 transcript levels was associated with enhanced T suppressor activity. To confirm that the expression of suppression markers was associated with the $CD4^+CD25^+$ T cells found in the tumor, the cells were further analyzed for Foxp3 and IL-10 expression by intracellular cytokine staining. Approximately 3% of intratumoral $CD4^+CD25^+$ T cells expressed Foxp3 and ~10% expressed IL-10 on day 0, demonstrating limited T suppressor cell presence in untreated tumors (Fig. 3*C*). The proportion of intratumoral $CD4^+CD25^+$ T cells expressing Foxp3 however, increased with each treatment (up to 43% on day 49), establishing that chronic stimulation resulted in a dramatic enhancement of T suppressor infiltration and/or expansion (Fig. 3*C*). To this end, quantitative analysis of data from multiple experiments established that the fraction of $CD4^+CD25^+Foxp3^+$ cells in TIL increased by >100-fold between days 0 and 49 (Table I). A similar increase in IL-10 expression by tumor-infiltrating $CD4^+CD25^+$ T cells (from 10 to 26%) was also observed following repeated treatment (Fig. 3*C*). Collectively, these data establish that the progressive increase in the expression of suppression markers during repeated treatment was the result of enhanced $CD4^+CD25^+$ T suppressor cell infiltration into tumors.

Enhanced T suppressor activity does not affect $CD8^+$ T cell IFN-γ production but impairs cytotoxic function

Previous studies (11) in our laboratory demonstrated that intratumoral IL-12 plus GM-CSF therapy induced a potent antitumor $CD8^+$ cytotoxic T cell response, which was required for tumor eradication. Although the above studies established that enhanced intratumoral Foxp3, IL-10, and TGFβ expression was associated with increased T suppressor activity, whether this resulted in the inhibition of antitumor CTL function was not determined. The initial finding that repeated treatment augmented intratumoral IFN-γ expression with increasing efficacy did not support this notion. To determine whether T cells were a significant source of IFN-γ, their ability to produce IFN-γ was analyzed by ELISPOT assay. Lymphocytes were isolated from tumors or the TDLN on days 0, 7, and 49, stimulated either with IL-2 and anti-CD3 Ab or a her-2/neu MHC class I peptide, and analyzed for IFN-γ production. The results are shown in Fig. 4. These data demonstrate that the ability of T cells to produce IFN-γ in response to anti-CD3 Ab stimulation did not diminish, but in fact increased with repeated treatment, and are consistent with the findings from the FNA/RT-PCR studies (Fig. 2*A*). Moreover, pulsing of cells with a her-2/neu MHC class I peptide also resulted in a progressive increase in IFN-γ-secretion (particularly within the TIL), suggesting that tumor-specific $CD8^+$ T cells were responsible for a significant portion of IFN-γ-production (Fig. 4). These data establish that repeated treatment augmented IFN-γ-production by T cells despite increased intratumoral T suppressor activity.

ELISPOT analysis provided strong evidence that increasing T-suppressor activity did not compromise T cell function in general, or intratumoral $CD8^+$ T cell activity in particular. To directly determine whether chronic stimulation enhanced the quantity and/or function of intratumoral $CD8^+$ T cells, we next monitored the $CD8^+$ T cell populations obtained from the TDLN and tumors during repeated treatment. Flow cytometric analysis of $CD8^+$ T cells showed no changes in the percentage of $CD8^+$ T cells on days 7 and 49 compared with day 0 in the TDLN (Fig. 5*A*). In contrast, the quantity of $CD8^+$ T cells within the TIL population increased by >3-fold between days 0 and 49 (Fig. 5*A*). To determine whether the increased T suppressor infiltration affected the ability of $CD8^+$ T cells to produce IFN-γ, $CD8^+$ T cells from the TDLN and TIL were analyzed by intracellular cytokine staining. The results demonstrate that treatment induced IFN-γ expression by both TDLN and tumor-infiltrating $CD8^+$ T cells with increasing efficacy after each injection, in agreement with the findings of FNA/RT-PCR and ELISPOT analyses (Fig. 5*B*). This increase was particularly dramatic within the tumor (~6-fold and ~22-fold increase in the proportion of IFN-γ-positive $CD8^+$ T cells on days 7 and 49, respectively). Collectively, these data demonstrate that treatment-induced T suppressor activity did not diminish the ability of $CD8^+$ T cells to infiltrate the tumors or produce IFN-γ in response to repeated therapy.

A recent study (20) showed that although T suppressor cells do not significantly impair the ability of CTL to produce cytokines in vivo, they effectively inhibit cytotoxic function through a TGFβ-dependent mechanism. To determine whether this was true in our model, $CD8^+$ T cells were isolated from the TDLN of mice on days 0, 7, and 49 and their ability to mediate tumor killing was tested in vitro (Fig. 5*C*). These results demonstrate that although the T cells isolated from day 0 mice mediated very little or no killing of tumor targets, a single treatment with IL-12 plus GM-CSF microspheres induced a dramatic increase in the ability of

Table I. *Fold increase in $Foxp3^+$ $CD4^+$ $CD25^+$ T-cells in tumors between days 0 and 49*[a]

	Percentage of TIL[a]			
	$CD4^+$	$CD4^+CD25^+$	$CD4^+CD25^+Foxp3^+$	Fold Change
Day 0	9.0 ± 3.4	0.20 ± 0.06	0.01 ± 0.005	1
Day 7	32.4 ± 6.8	1.63 ± 0.44	0.42 ± 0.04	42
Day 49	40.4 ± 7.4	2.51 ± 1.11	1.09 ± 0.48	109

[a] Data are averages of values from three independent experiments. Error = SD.

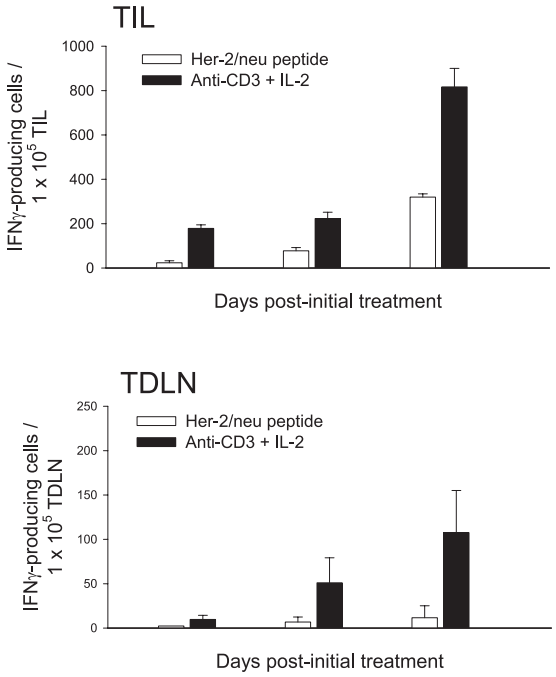

FIGURE 4. ELISPOT analysis of IFN-γ production by T cells during repeated treatment. Cells were isolated from tumors (TIL) and the TDLN and analyzed for IFN-γ production by ELISPOT assay on days 0 (pretreatment), 7 (1 wk after the first treatment), and 49 (1 wk after the third treatment) as described in *Materials and Methods*. Cells from three mice were pooled and processed in duplicate for each time point. In the TIL study, the differences between all time points were significant ($p < 0.048$) for the her-2/neu peptide group. In the anti-CD3/IL-2 group, differences between days 0 vs 49 and 7 vs 49 were significant ($p < 0.0007$). In the TDLN study, the difference between days 0 and 49 was significant in the anti-CD3/IL-2 group ($p = 0.012$). Error bars, SD.

$CD8^+$ T cell populations isolated (on day 7) from the TDLN to lyse tumor cells. In contrast, after three successive treatments, day 49 $CD8^+$ T cells demonstrated a significant reduction in cytotoxic function. These data establish that the progressive increase in the quantity of intratumoral T suppressor cells is associated with a concurrent decrease in the ability of cytotoxic $CD8^+$ T cells to mediate tumor killing.

Loss of $CD8^+$ T cell cytotoxic function is accompanied with deletion of tumor-specific CTLs

The studies above focused on the total $CD8^+$ T cell population but did not investigate their tumor specificity. It has been shown (21) that her-2/neu, which is overexpressed by tumors in this strain represents a bona fide tumor Ag that can be recognized by CTL. More recently (16), a MHC class I epitope was identified for rat her-2/neu. To determine whether IL-12 plus GM-CSF microsphere treatment induced her-2/neu-specific $CD8^+$ T cells and to establish how these cells responded to repeated treatment, tetramer analysis of her-2/neu-specific $CD8^+$ T cells was performed. Treatments were administered on days 0, 21, and 42, cells were isolated from the tumors and the TDLN on days 0 (before therapy), 7, and 49, and tetramer-specific $CD8^+$ T cells were quantified by flow cytometry (Fig. 6). Analysis of tumor-infiltrating $CD8^+$ T cells in untreated tumor-bearing mice and splenic $CD8^+$ T cells of nontumor-bearing control mice demonstrated identical levels of tetramer-positive cells, suggesting that the mice were tolerant to her-2/neu (Fig. 6, Control). In contrast, a single injection of IL-12 plus GM-CSF microspheres induced a dramatic (>12-fold) increase in the quantity of her-2/neu-specific $CD8^+$ T cells in the tumor (from 3 to 36%) confirming that intratumoral IL-12 plus GM-CSF promotes a potent CTL response that infiltrates tumors successfully (Fig. 6, TIL). A significant 4-fold increase in tetramer-positive cells is also observed in the TDLN on day 7 (Fig. 6, TDLN). More importantly, however, following repeated treatment, a 30-fold reduction in the quantity of tetramer-specific $CD8^+$ T cells is observed in the tumor on day 49 in comparison to day 7 (Fig. 6, TIL). These findings demonstrate that the reduction in the cytotoxic function of the $CD8^+$ T cell component is paralleled with extensive changes in the clonal composition of $CD8^+$ T cell populations in the long term.

Beause increased intratumoral T suppressor activity did not appear to affect the ability of $CD8^+$ T cells to produce IFN-γ, we wanted to determine whether this was true for tumor-specific $CD8^+$ T cells as well. $CD8^+$ T cells obtained from tumors on days 0, 7, and 49 were stained for intracellular IFN-γ and analyzed by flow cytometry. The results demonstrate that although the quantity of tetramer-specific T effectors declined over the long term, the remaining cells (on day 49) still responded very strongly to IL-12 plus GM-CSF treatment, with a significantly higher ratio of tetramer-specific cells producing IFN-γ compared with their day 7 counterparts (Fig. 6, TIL IFN-γ-production). This finding essentially mirrored the pattern displayed by the total $CD8^+$ T cell population, further confirming the lack of correlation between the intensity of IFN-γ response and the antitumor efficacy of $CD8^+$ T cells in the repeated vaccination setting.

Discussion

These studies demonstrate that the failure of chronic immune therapy to maintain tumor suppression in her-2/neu transgenic mice is not due to an inability to induce immune activation, but rather is associated with the development of an increasingly stronger intratumoral T suppressor cell response, resulting in enhanced blocking of T cell cytotoxicity. Furthermore, augmentation of homeostatic regulatory activity is accompanied with a dramatic loss of tumor-specific $CD8^+$ CTL from the tumor microenvironment in the long term. Thus, these data provide important insights into the question of why repeated vaccination, while able to enhance peripheral antitumor immunity, fails to induce effective tumor eradication.

Quantitative analysis of immune activation and suppression markers in posttherapy tumors demonstrated that intratumoral delivery of IL-12 and GM-CSF promoted a rapid reversal of the suppressive characteristics of the tumor microenvironment. More specifically, treatment enhanced intratumoral IFN-γ production and suppressed Foxp3, TGFβ, and IL-10 expression. The ability of IL-12 to induce IFN-γ-production by T, NK, and NKT cells is well established (22). Our studies confirmed that augmentation of intratumoral IFN-γ-expression was due, at least in part, to enhanced $CD8^+$ T cell infiltration. The mechanism(s) underlying the reduction in Foxp3, TGFβ, and IL-10 expression, however, remain to be elucidated. IFN-γ has been shown to inhibit the generation and activation of $CD4^+CD25^+$ T suppressor cells (23). Therefore, it is possible that IFN-γ directly mediated the suppression of TGFβ and IL-10 in the above studies. In contrast, the robust recovery of both TGFβ and IL-10 on day 7 despite continued high levels of IFN-γ does not support this notion. Alternatively, the treatment-induced decline in Foxp3, IL-10, and TGFβ expression could be due to a quantitative loss of $CD4^+CD25^+$ T cells from the tumor microenvironment and/or a change in their functional characteristics. To this end, we are currently characterizing the posttherapy quantitative and qualitative changes in the tumor-infiltrating $CD4^+$ $CD25^+$ T suppressor population.

Another important finding in this study was that the reversal of intratumoral immune suppression was transient. In fact, T suppressor

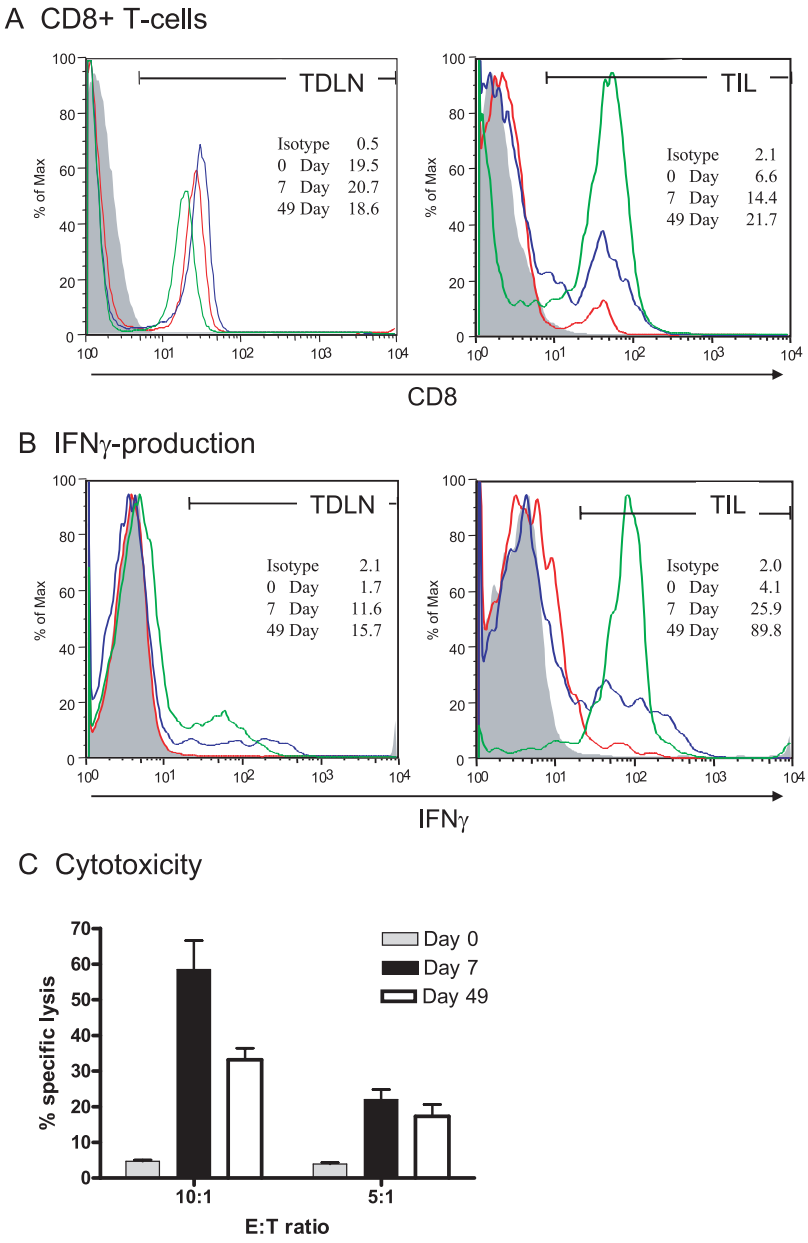

FIGURE 5. Effect of repeated treatment on intratumoral CD8$^+$ T cell activity. The ability of CD8$^+$ T cells isolated from the TDLN and tumors (TIL) to produce IFN-γ and mediate cytotoxic killing were evaluated. *A*, The proportion of CD8$^+$ T cells within the TDLN or TIL populations was quantified during repeated treatment. *B*, Percent IFN-γ-positive cells within the CD8$^+$ T cell population. CD8$^+$ T cells were gated on and analyzed for IFN-γ expression by intracellular staining. The results shown for *A* and *B* are representative of at least two, in some cases three, independent experiments. Gray shading, isotype control; red, day 0; blue, day 7; and green, day 49. *C*, Cytotoxic killing of her-2/neu-expressing tumors by CD8$^+$ T cells before treatment (day 0), 1 wk after the first treatment (day 7), and 1 wk after the third treatment (day 49). Cells were pooled from the TDLN of three mice per group. Combined data from two experiments are shown. The differences between days 0 and 7 and days 7 and 49 were statistically significant ($p = 0.0001$ and 0.037, respectively) at the E:T ratio of 10:1. Error bars, SD.

activity recovered within 1 wk of treatment as indicated by the rebound in intratumoral Foxp3, TGFβ, and IL-10 mRNA levels. This increase was associated with an upsurge in the numbers of tumor-infiltrating CD4$^+$CD25$^+$Foxp3$^+$ T suppressor cells. The rapid resurgence of suppressor mechanisms between days 4 and 7 indicated that recovery of regulatory activity was tightly linked to effector CD8$^+$ T cell development and that the antitumor CTL had a narrow window of opportunity for eliminating tumors. These findings are consistent with the results of a recent study (24), in which Ag-specific vaccination of tumor-bearing mice resulted in the systemic amplification of adoptively transferred Ag-specific CD4$^+$CD25$^+$Foxp3$^+$ cells. Collectively, these observations support the notion that amplification of T suppressor cells is a critical and highly effective mechanism for homeostatic regulation of posttreatment immune activity within tumors. In contrast, how immune activation leads to a rapid expansion of T suppressors in tumors remains to be determined.

Repeated treatment was able to reverse resurgent suppressor activity; however, this reversion occurred with decreasing efficacy following each treatment. The loss of efficacy correlated with an intensification of the T suppressor rebound. These results correlate well with our previous findings (11), in which repeated treatment resulted in diminished antitumor efficacy in this model. In our previous study, we could not rule out the possibility that the loss of antitumor efficacy was due to a change in the antigenic profile of the tumor. To this end, analysis of her-2/neu expression, which was shown above to be a bona fide target for CTL, did not demonstrate a significant loss of her-2/neu from tumors during repeated treatment (data not shown). These findings are consistent with the hypothesis that the observed loss of therapeutic efficacy during chronic immune stimulation is mediated by a progressive enhancement of the homeostatic regulatory mechanisms rather than the loss of tumor Ag in this model.

Previous studies (22), in numerous laboratories including ours, had established the critical role of IFN-γ in IL-12-mediated tumor eradication providing the rationale for our selection of IFN-γ as a marker for intratumoral immune activation. However, the finding that repeated treatment augmented intratumoral IFN-γ-production but not tumor suppression demonstrated that IFN-γ alone was not sufficient for tumor eradication. This is consistent with the results from a previous report (25), which demonstrated a lack of correlation between intratumoral IFN-γ levels and tumor regression in

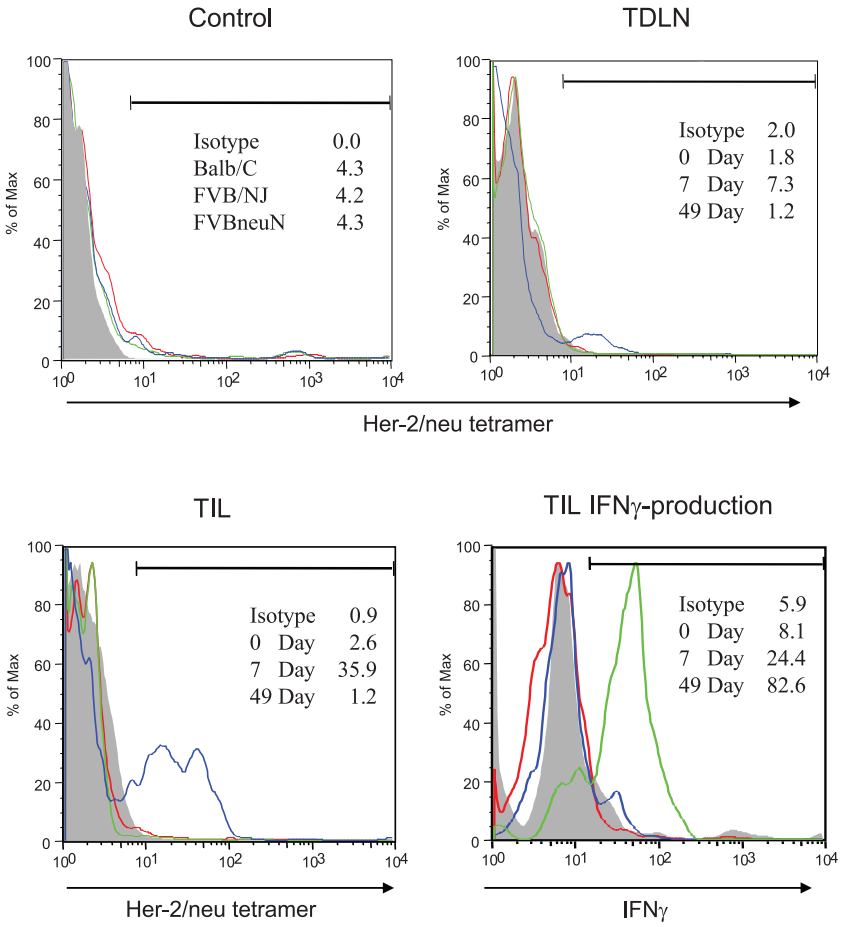

FIGURE 6. Induction of her-2/neu-specific cytotoxic T cells by IL-12 plus GM-CSF microsphere treatment. Control, The presence of her-2/neu-specific $CD8^+$ T cells in the spleens of BALB/c, wild-type FVB/NJ, and tumor-bearing untreated FVB-neuN mice were evaluated by a tetramer-binding assay. TDLN, Cells were isolated from the TDLN of tumor-bearing FVBneuN mice before treatment (day 0), 1 wk after the first treatment (day 7), and 1 wk after the third treatment (day 49). $CD8^+$ T cells were gated on and analyzed for tetramer binding. TIL, Single-cell suspensions were prepared from the tumors of FVBneuN mice on indicated days, $CD8^+$ T cells were gated on and analyzed for tetramer binding. TIL IFN-γ production, Single-cell suspensions obtained from tumors on indicated days were stained for CD8, tetramer binding, and intracellular IFN-γ. Double-positive cells (CD8 and tetramer) were gated on and evaluated for IFN-γ production. All data (except control) are representative of two independent experiments.

melanoma patients undergoing peptide vaccination. Furthermore, the above results did not demonstrate a direct antagonistic relationship between intratumoral IFN-γ levels and T suppressor activity, suggesting that intratumoral T suppressor cells were mediating their effect through a mechanism that did not involve suppression of IFN-γ. A recent report (20) showed that T suppressor cells do not necessarily inhibit cytokine production by $CD8^+$ T effectors but mediate their effects via direct suppression of cytotoxic function in a TGFβ-dependent manner in vivo. Consistent with these findings, analysis of T cell cytotoxic function after one and three treatments demonstrated that multiple treatments resulted in reduced cytotoxic function despite enhanced IFN-γ production. Although T suppressor cells were identified as the most likely instigator of CTL dysfunction in this model, the unusually high levels of intratumoral IFN-γ (>9000-fold above pretherapy) observed after the third treatment raised the possibility that this cytokine itself could be directly involved in T cell inactivation. IFN-γ has been shown (26) to induce the production of IDO by APCs, an enzyme that can mediate T cell anergy via tryptophan catalysis. Therefore, it was also possible that enhanced IFNγ production resulted in IDO-mediated CTL anergy independent of T suppressor activity. Preliminary analysis of posttherapy tumors demonstrated a 5- to 10-fold increase in IDO transcript levels between 6 and 72 h, supporting this notion (data not shown). Recently, T suppressor cells were also shown to induce IDO production by dendritic cells via CTLA-4 ligation (27). Whether enhanced IDO expression was due specifically to IFN-γ, to T suppressor cells, or both, and whether IDO contributed significantly to the loss of CTL cytotoxicity, remains to be determined.

Further analysis of intratumoral CTL activity using tetramer analysis demonstrated a major shift in the clonal profile of the CTL during repeated therapy. Whereas the initial treatment successfully promoted the infiltration of tumors with her-2/neu-specific $CD8^+$ T cells comprising >35% of all $CD8^+$ T cells, the proportion of these cells declined dramatically (<2%) after repeated therapy. Persistent exposure to Ag results in exhaustion/deletion of Ag-specific $CD8^+$ T cells in both chronic virus infection and established tumor models (28–30). The observation that repeated therapy results in the loss of her-2/neu-specific $CD8^+$ T cells is consistent with Ag overload-mediated exhaustion because her-2/neu expression on tumors was not altered during long-term therapy. Repeated stimulation with cytokine-encapsulated microspheres may in fact accelerate this process. One unanswered question in this study is whether the observed loss of $CD8^+$ T cell cytotoxicity in chronically treated mice is due to enhanced T suppressor activity and/or IDO, to the loss of dominant CTL, or a combination of all of these factors. Studies addressing this question are currently underway in our laboratory. Another interesting question is whether the two mechanisms identified in this study, i.e., augmentation of T suppressor activity and clonal deletion, are independent events or whether intensification of the suppressor response contributes to the loss of her-2/neu-specific CTL from the tumor since T suppressor cells have been shown to possess cytotoxic potential (31). Current evidence from numerous in vitro and in vivo studies (32) does not support a direct role for T suppressors in the control of CTL survival, suggesting that suppression and exhaustion are distinct but complementary events.

The above findings have important clinical implications for the specific approach tested in this study and for cancer immune therapy in general. First, the results demonstrate the importance of the tumor microenvironment in posttherapy immune monitoring as both $CD8^+$ T cells and T suppressors rapidly home to tumors following immune activation. Second, the data establish that posttreatment T cell IFN-γ

secretion, a parameter that is commonly used to monitor patient immunity, does not correlate with the efficacy of tumor kill and that monitoring of granzyme/perforin secretion patterns of intratumoral (or TDLN) T cells will likely provide a better correlate. Finally, our studies demonstrate that induction of antitumor immunity is rapidly counteracted by homeostatic regulation, and that repeated stimulation results in a progressive loss of therapeutic efficacy due to increased suppressor activity and eventual immune exhaustion. This finding suggests that standard vaccination protocols have a limited window of efficacy in the established disease setting. Studies (33–35) have shown that this window can be broadened by blocking homeostatic regulation. On the other hand, although blocking of regulatory mechanisms can dramatically enhance effector activity, this approach does not address the longer-term immune exhaustion issue. Clonal deletion can particularly become a drawback in patients with bulky and persistent disease who require chronic treatment. Therefore, in addition to modulation of immune regulatory mechanisms, approaches targeting multiple Ags in patients with minimal residual disease are likely to provide a more effective vaccine strategy in cancer patients (36).

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Disclosures

The authors have no financial conflict of interest.

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